AD_____

GRANT NUMBER DAMD17-96-1-6163

TITLE: New Approaches to Chemoprevention of Breast Cancer

PRINCIPAL INVESTIGATOR: Michael B. Sporn, Ph.D.

CONTRACTING ORGANIZATION: Dartmouth College
Hanover, NH 03755

REPORT DATE: September 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Final (1 Sep 96 - 31 Aug 98)

4. TITLE AND SUBTITLE
New Approaches to Chemoprevention of Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6163

6. AUTHOR(S)
Michael B. Sporn, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Dartmouth College
Hanover, NH 03755

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Triterpenoids, natural products related to steroids and retinoids, represent an important class of new structures for drug discovery, with potential applications in many fields of medicine, particularly cancer. This project involves the development of new synthetic triterpenoids for eventual use as agents for chemoprevention or chemotherapy of breast cancer. Although the naturally occurring triterpenoids, ursolic acid (UA) and oleanolic acid (OA), have been shown to have some anti-carcinogenic activity, they are relatively weak agents. During the past year, we have synthesized over 150 new triterpenoids, and many of these have been assayed as inhibitors of de novo formation of inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2), two enzymes highly relevant to the carcinogenesis in the breast. We have also screened these new triterpenoids as inducers of differentiation in NB-4 leukemia cells and as non-cytotoxic suppressors of estrogen-stimulated growth in MCF-7 breast cancer cells. Several new triterpenoids are markedly more active in these assays than their respective parents, UA or OA.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	42
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

WBS For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

WBS In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

WBS In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Michael B. Spinn, M.D. 9/29/98

PI - Signature Date

Table of Contents

(1) Front Cover	1
(2) Standard Form 298	2
(3) Foreword	3
(4) Table of Contents	4
(5) Introduction	5
(6) Body	6 - 21
(7) Conclusion	22
(8) References	23 - 24
(9) Appendices	25

(5) INTRODUCTION

There is a major need for new drug discovery in the field of breast cancer, and this project is directed toward that goal. There is a particular need for development of new agents that will inhibit progression of premalignant and early malignant lesions to more aggressive, invasive and metastatic stages, since screening techniques are now identifying large numbers of women with such early breast lesions. Furthermore, advances in genetic testing are leading to the identification of many women with a high risk for future development of breast cancer, for whom at present there is no satisfactory preventive modality.

Triterpenoids of an ursane or oleanane structure are very widely distributed in nature, occurring in hundreds of plants all over the world. Although triterpenoids are widely used for medicinal purposes in many Asian countries, this class of molecules, which resemble steroids in their chemical structure, biogenesis, and pleiotropic actions, has not impacted on the practice of Western medicine. Triterpenoids, like the steroids, are formed in nature by the cyclization of squalene, with the retention of all 30 carbon atoms in molecules such as oleanolic acid (OA) and ursolic acid (UA). Although OA and UA are known to have numerous pharmacological activities, including chemoprevention of cancer in experimental animals (Nishino et al., 1988; Huang et al., 1994), the potency of these naturally occurring molecules is relatively weak. Chemical synthesis of new steroid analogs has provided many useful derivatives that are more potent and specific than natural parent structures. With this as a model, and considering the known anti-carcinogenic activities of OA and UA, we have started a new project to synthesize and characterize a new series of synthetic triterpenoid analogs as potential inhibitors of mammary carcinogenesis, using suppression of the formation of nitric oxide and prostaglandins, as well as induction of cadherins/catenins, as assay systems. In addition to these assay systems, we have also performed preliminary assays on new triterpenoids as non-cytotoxic inhibitors of DNA synthesis in human MCF-7 breast cancer cells.

The inducible enzymes that mediate the formation of nitric oxide and prostaglandins (iNOS and COX-2, respectively) are now the focus of major interest in carcinogenesis studies. Elevated activity of both of these enzymes has been particularly implicated in colon carcinogenesis (Takahashi et al., 1997; Prescott and White, 1996), but there is also evidence for their causative involvement in breast cancer (Thomsen et al., 1995; Liu and Rose 1996). Extensive data exist for the role of the cadherin/catenin system in breast cancer (Anzano et al., 1994).

(6) BODY

a) Experimental Methods

1. Studies on Human Breast Cancer Cells

Cell Maintenance:

MCF-7, T47D, or SK-Br-3 cells were maintained in DMEM/F12 with phenol red, 10% fetal bovine serum (Hyclone), Pen/Strep, in a 37°C, 5% CO_2 humidified incubator.

Treatment for Experiment:

Cells were harvested by trypsinization, resuspended in experimental media (RPMI without phenol red, 10% charcoal/dextran-stripped FBS (Hyclone), Pen/Strep), sedimented and washed once with the same media. Cells were then seeded in experimental media at 1200 cells per well in 96-well plates for MTT assay, 6000 cells per well in 24-well plates for ^{3}H-thymidine incorporation, or 10^6 cells per 9-cm dish for RNA extraction.

Addition of reagents:

Equal volume of experimental media containing 17 β-estradiol (final concentration = 10 pM), desired triterpenoid compound dissolved in DMSO, or vehicle alone at final concentration = 0.1% was added to the cells. Unstimulated control wells received vehicle in experimental media without 17 β-estradiol. Cells were incubated in compounds for three days (^{3}H-thymidine incorporation and RNA extraction) or five days (MTT assay).

Assays

1)MTT

1/10 volume of 5mg/ml MTT (Sigma) in experimental media was added to the cells. After 3-4 hours incubation at 37°, the media was aspirated and 100 µl of DMSO was added to each well to solubilize the dye. Absorbance at 570 nm was read using a microtiter plate reader.

2)Thymidine incorporation

5 µCi ^{3}H-thymidine was added to each well. After two hours incorporation time, the media was aspirated, the wells were washed, and the monolayer was fixed with 10% TCA. Nucleic acids were then solubilized with 0.2 N NaOH, 40 µg/ml salmon sperm DNA, and incorporated ^{3}H was measured.

3)Northern blot

Total RNA was extracted using the TRIzol method (Life Technologies) and run on a MOPS-agarose gel with 1.85% formaldehyde. RNA was transferred to a nylon membrane, cross-linked, and hybridized to ^{32}P-labeled probes for two days.

4)Western blot

Total protein was extracted from cells after exposure to compounds for three days. Equal amounts of protein (based on BCA assay) were loaded on polyacrylamide gels and transferred to nitrocellulose. The membranes were incubated with β-catenin antibodies (Transduction Laboratories). Detection was by chemiluminescence using Amersham ECL reagents, and films were scanned by densitometry.

2. Studies on Macrophages

Full details of methods for cell culture of primary mouse macrophages and the macrophage-like cell line, RAW 264.7, are given in the attached article, "Novel Triterpenoids Suppress Inducible Nitric Oxide Synthase (iNOS) and Inducible Cyclooxygenase (COX-2) in Mouse Macrophages," by Nanjoo Suh, Tadashi Honda, Heather Finlay, Aaaron Barchowsky, Charlotte Williams, Nicole Benoit, Qiao-wen Xie, Carl Nathan, Gordon W. Gribble, and Michael B. Sporn, (Cancer Res. 58: 717-723, 1998) which describes the suppression of de novo formation of iNOS and COX-2 by synthetic triterpenoids made with support from this grant. Likewise, methods for assay of mRNA, protein, and enzyme product for bothiNOS and COX-2 are presented in detail in this manuscript.

b) Results and Discussion

1. Synthesis of New Triterpenoids

The synthesis of new triterpenoid derivatives of oleanolic and ursolic acids is described in detail in the attached articles, "New Enone Derivatives of Oleanolic Acid and Ursolic Acid as Inhibitors of Nitric Oxide Production in Mouse Macrophages," by Tadashi Honda, Heather Finlay, Gordon Gribble, Nanjoo Suh, and Michael B. Sporn, published in Bioorganic & Medicinal Chemistry Letters 7: 1623-1628, 1997. Structures of other triterpenoids reported here are shown in Figure 1, and the attached preprint, "Design and Synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28oic acid, A Novel and Highly Active Inhibitor of Nitric Oxide Production in Mouse Macrophages", by Tadashi Honda, BarbieAnn V. Rounds, Gordon W. Gribble, Nanjoo Suh, Yongping Wang and Michael B. Sporn, in Bioorganic & Medicinal Chemistry Letters in press (1998).

2. Results with Human Breast Cancer Cells

Suppression of DNA synthesis in MCF-7 human breast cancer cells by 7 triterpenoids, without evident cytotoxicity, is shown in Figure 2. At this time, there is no apparent set of structure-activity relationships. Studies on modulation of ß-catenin expression have been pursued in SK-Br-3 cells. Figure 3 shows that all-trans-retinoic acid is a potent inducer of ß-catenin expression in these cells, as measured by Western blot analysis after 3 days of treatment. However, we have yet to see a strong inductive effect on ß-catenin with any triterpenoid that we have tested so far. In fact, as shown in Figure 4, 3-keto-oleanolic acid (3-keto-OA) and 3-epi-ursolic acid (3-epi-UA), when tested at 10

micromolar, appear to have an inhibitory effect on ß-catenin expression; furthermore, these two triterpenoids appear to block the stimulatory effect of all-trans-retinoic acid. In contrast, as shown in Figure 4, 3-epi-oleanolic acid (3-epi-OA) has a slight stimulatory activity on ß-catenin expression when tested at 10 micromolar. A large number of new triterpenoids remain to be tested in this assay system. One other significant observation that we have made is shown in Figure 5. TP-82, which was shown in Figure 2 as a potent suppressor of DNA synthesis in MCF-7 cells, is shown in Figure 5 to downregulate the expression of the estrogen receptor (ER-alpha) in these cells. Since MCF-7 cells are known to be ER-positive, and the growth of these cells is known to be driven by 17-ß-estradiol, this suppression of the estrogen receptor may account, at least in part, for the growth-suppressive activity of TP-82 in the MCF-7 cells. Figure 6 shows the extremely high potency of the new triterpenoid, CDDO (TP-151) in blocking growth of MCF-7 cells (ER-positive). Figures 7-12 show inhibitory effects of TP-151 on various ER-negative breast cancer cell lines.

3. Results with Macrophages

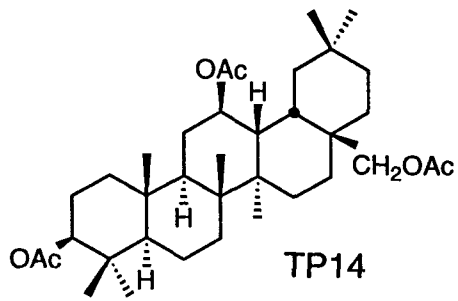
The ability of triterpenoids to suppress de novo formation of two enzymes, inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) is most easily measured in macrophages, using either primary mouse peritoneal macrophages or a mouse macrophage-like cell line (RAW 264.7) as assay systems. The important relevance of iNOS and COX-2 for carcinogenesis (including carcinogenesis in the breast) is discussed in the Introduction. Using either gamma-interferon or lipopolysaccharide (LPS) as inducing agents, we can achieve major inductions of de novo synthesis of both iNOS and COX-2 in the above cells. Two synthetic oleananes, 3,12-dioxoolean-1-en-28-oic acid (TP-69), and 3,11-diooxoolean-1,12-dien-28-oic acid (TP-72) have been shown to be highly active inhibitors of these inductions; the attached article, "Novel Triterpenoids Suppress Inducible Nitric Oxide Synthase (iNOS) and Inducible Cyclooxygenase (COX-2) in Mouse Macrophages," by Nanjoo Suh, Tadashi Honda, Heather Finlay, Aaron Barchowsky, Charlotte Williams, Nicole Benoit, Qiao-wen Xie, Carl Nathan, Gordon Gribble, and Michael Sporn, documents these findings in detail. These data all suggest that further studies on the ability of triterpenoids to suppress iNOS and COX-2 should be pursued, and that we should continue the chemical synthesis and testing program that we have outlined above.

4. Results with Induction of Differentiation in Human Leukemia Cells

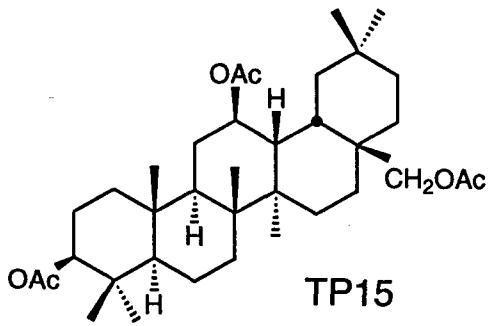
We have used the human cell line NB-4 in preference to HL-60, since NB-4 is a true promyelocytic leukemia. Induction of differentiation in NB-4 cells by TP-82, and in synergy with 9-cis-retinoic acid, is shown in Figure 13.

Figure 1

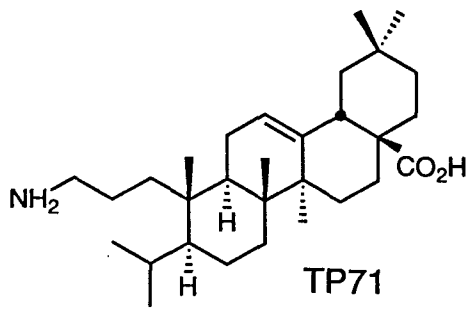
Structures of Growth-Inhibitory Triterpenoids



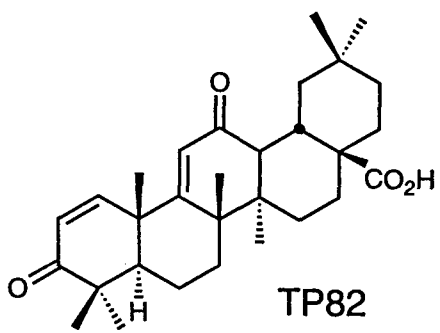
TP14



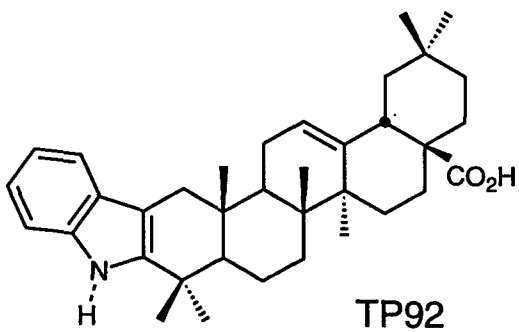
TP15



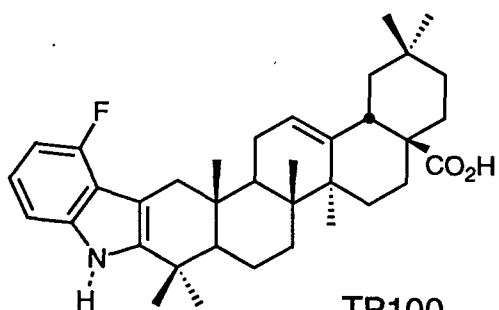
TP71



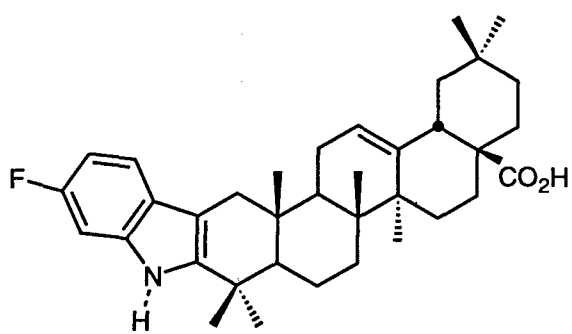
TP82



TP92



TP100



TP101

Figure 2

Suppression of DNA Synthesis in MCF-7 Cells by Triterpenoids

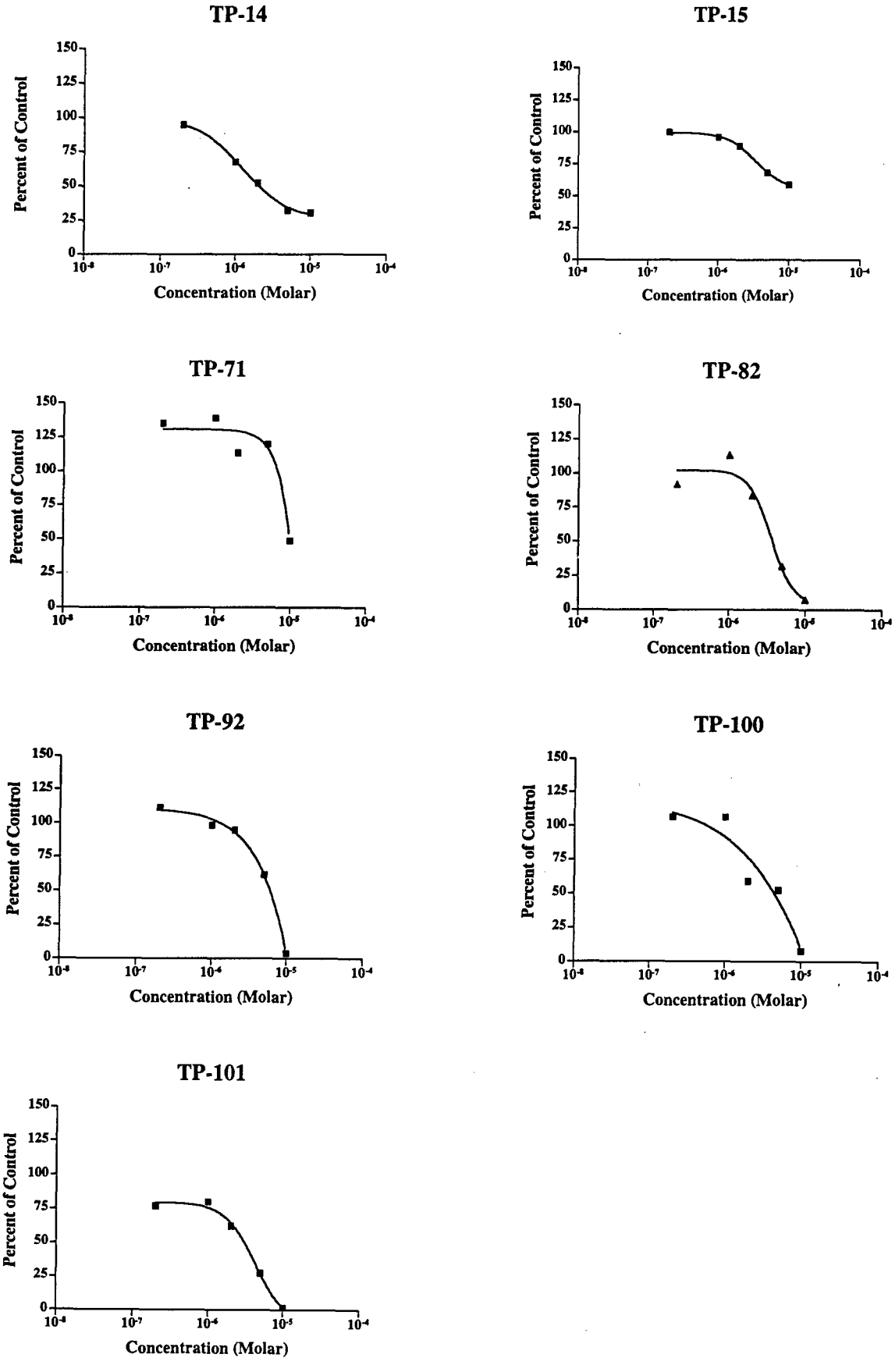


Figure 3

Induction of β-Catenin Expression in SK-Br-3 Cells

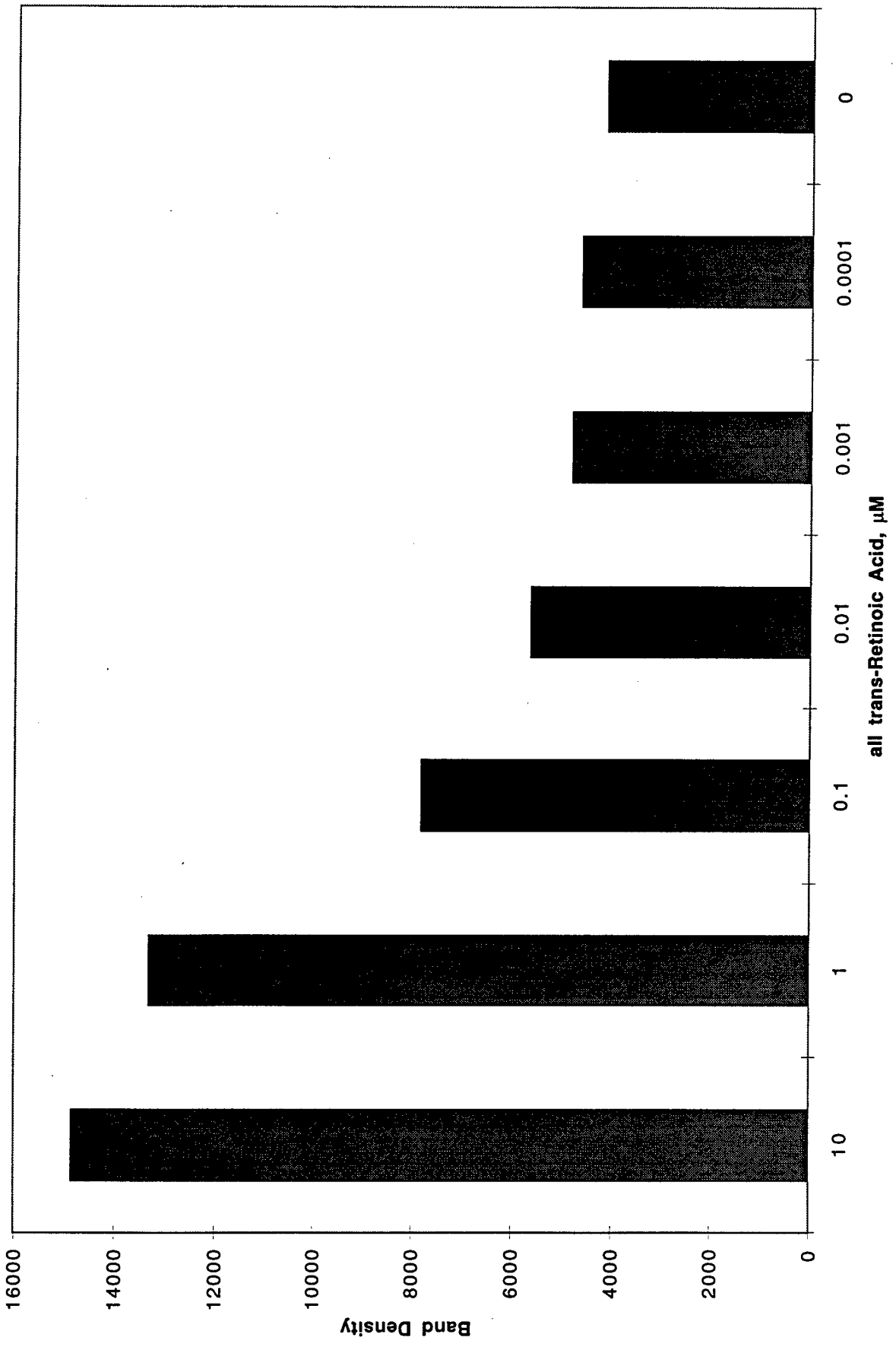


Figure 4

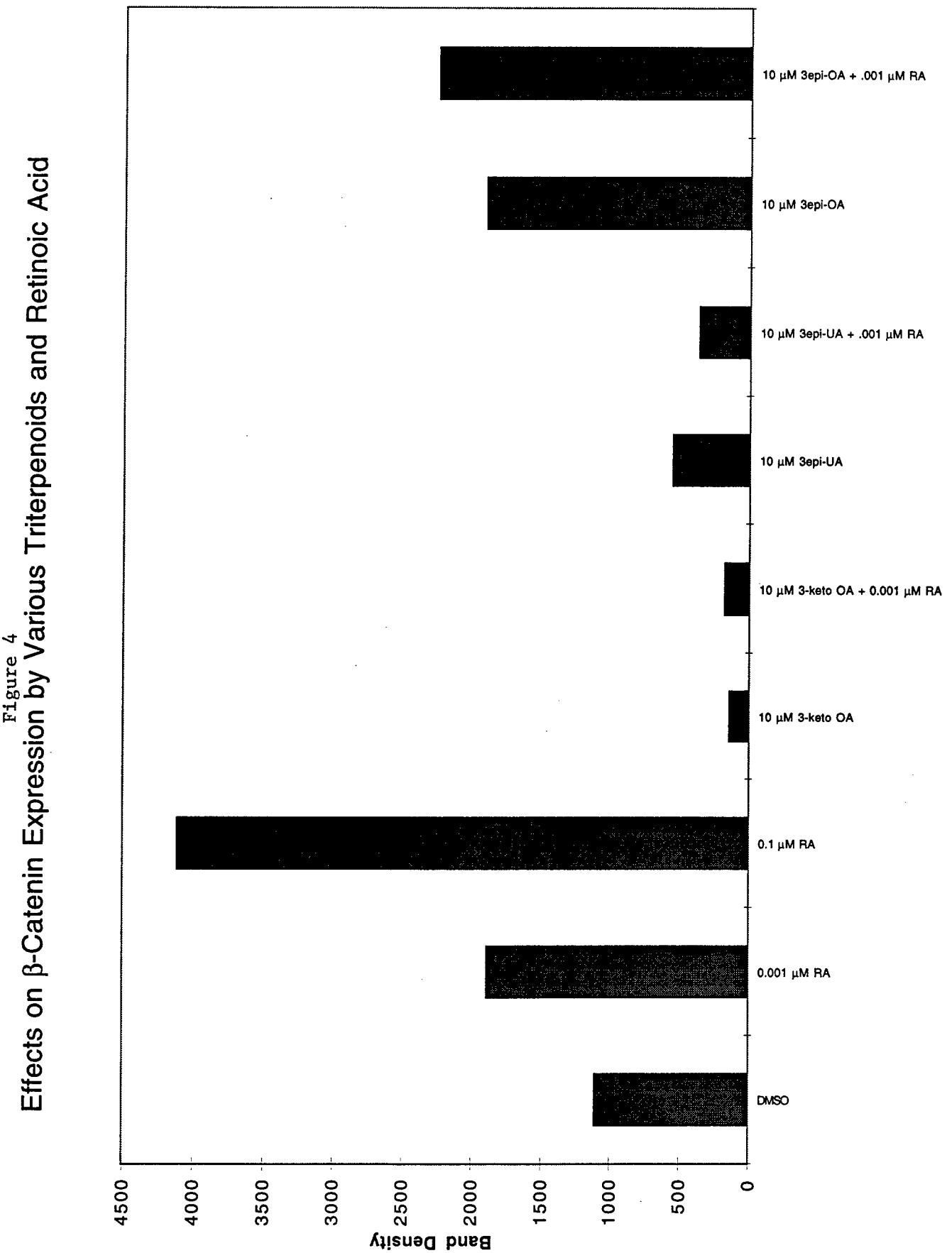

Effects on β-Catenin Expression by Various Triterpenoids and Retinoic Acid
Band Density
4500
4000
3500
3000
2500
2000
1500
1000
500
0
10 μM 3epi-OA + .001 μM RA
10 μM 3epi-OA
10 μM 3epi-UA + .001 μM RA
10 μM 3epi-UA
10 μM 3-keto OA + 0.001 μM RA
10 μM 3-keto OA
0.1 μM RA
0.001 μM RA
DMSO

Figure 5

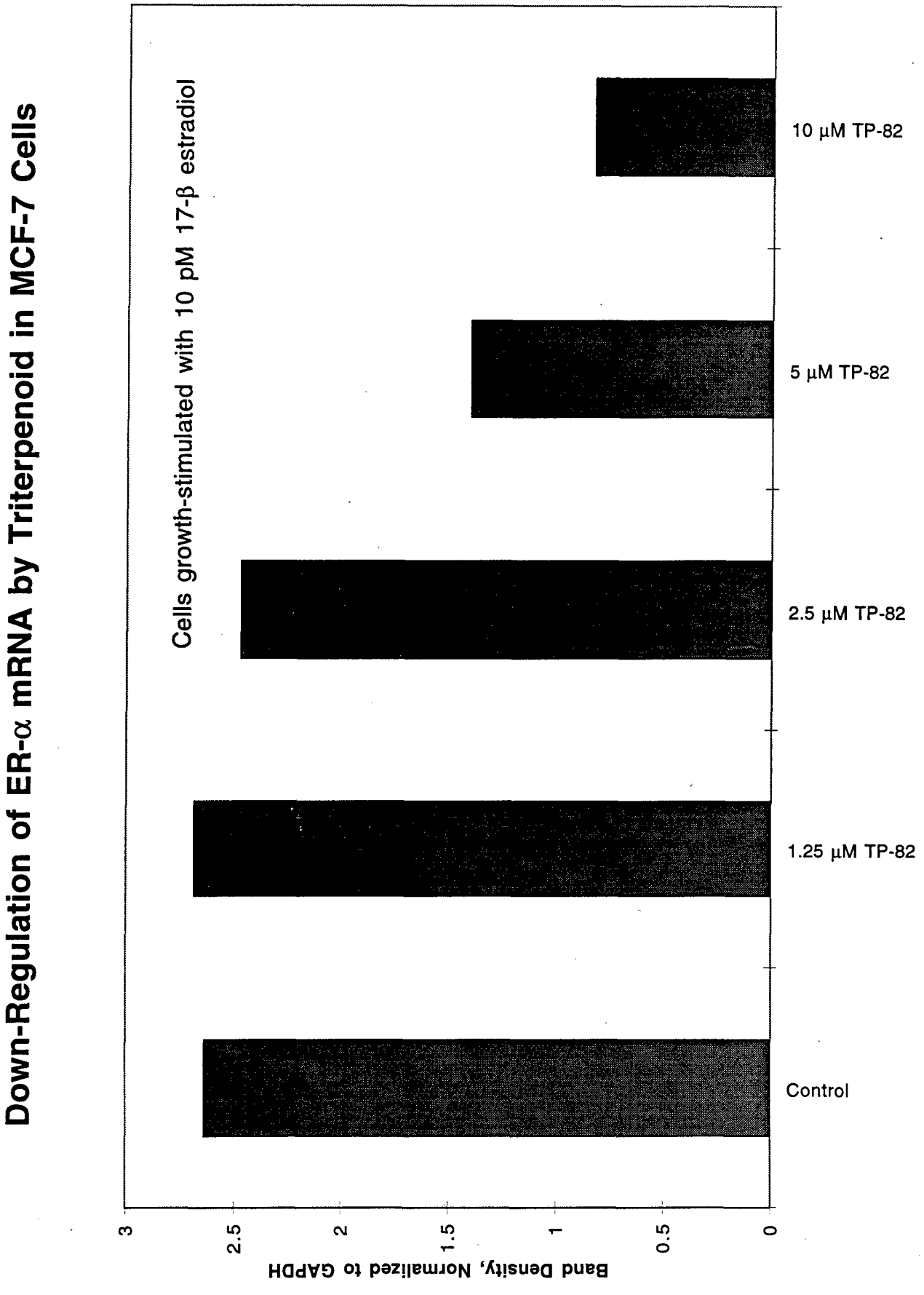
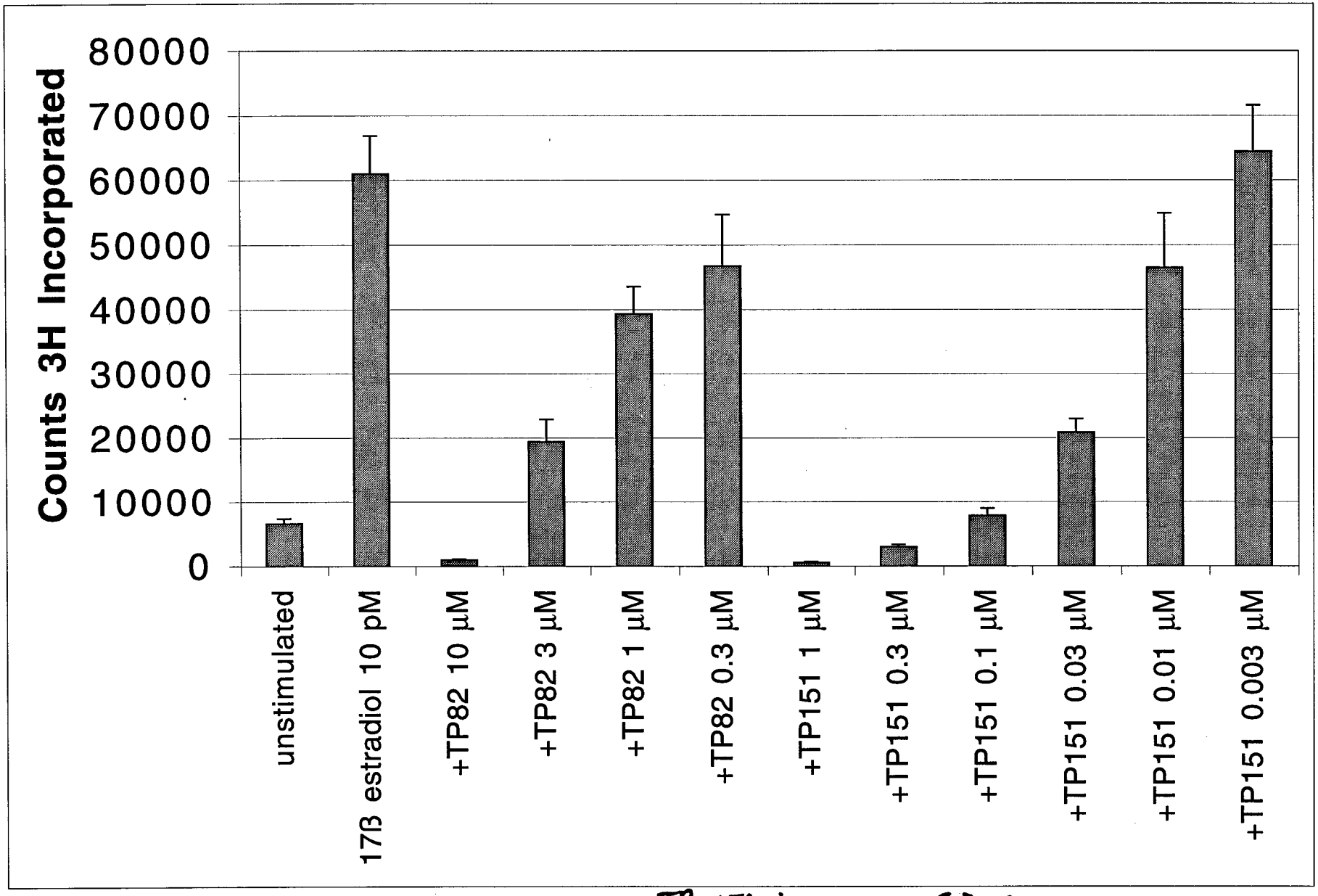

Down-Regulation of ER-α mRNA by Triterpenoid in MCF-7 Cells
Cells growth-stimulated with 10 pM 17-β estradiol
10 μM TP-82
5 μM TP-82
2.5 μM TP-82
1.25 μM TP-82
Control
Band Density, Normalized to GAPDH
0
0.5
1
1.5
2
2.5
3

Figure 6

Inhibition of Estrogen-Stimulated Growth by Triterpenoids in MCF-7 Breast Cancer Cells (ER-Positive)

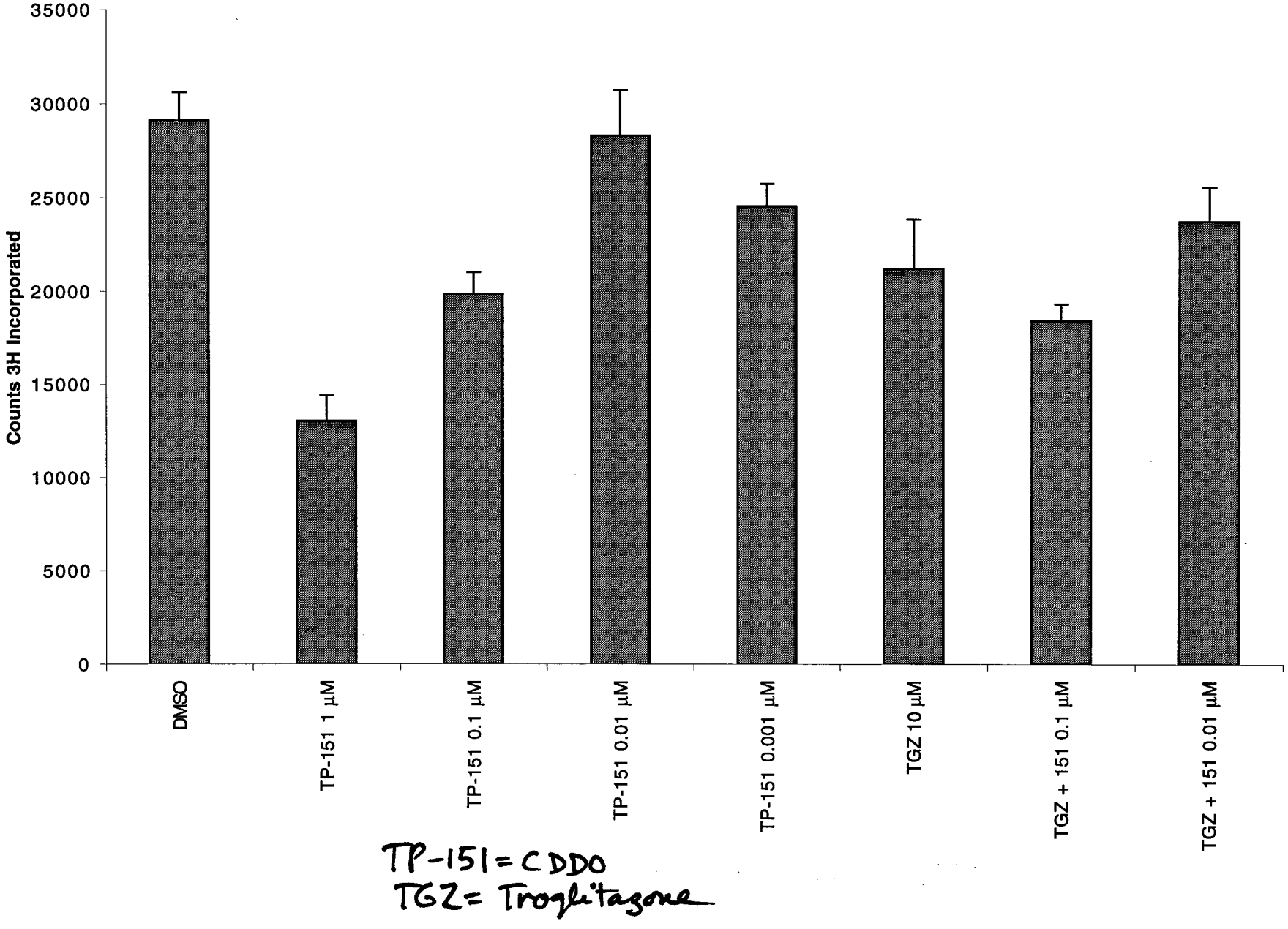


10% charcoal-stripped FBS, phenol red-free RPMI
72 hours treatment with compounds and 10 pM 17β estradiol
2 hours thymidine pulse

TP-151 is same as CDDO

Figure 7

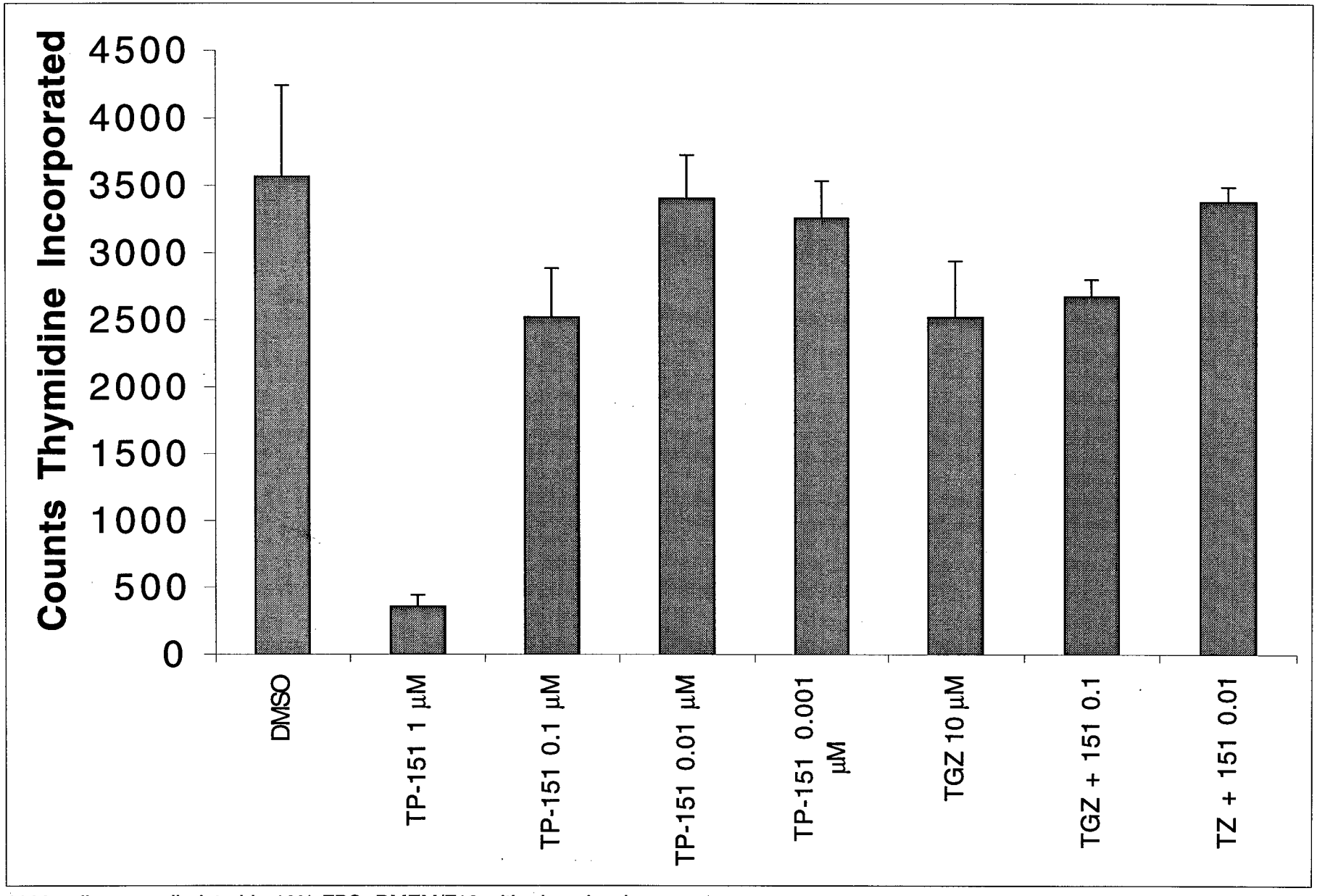
Growth Inhibition of MDA-231 ER-Negative Breast Cancer Cells



TP-151 = CDDO
TGZ = Troglitazone

Figure 8

MDA-468 ER Neg Breast Cancer Cells: Growth Effects by TP-151 and TGZ

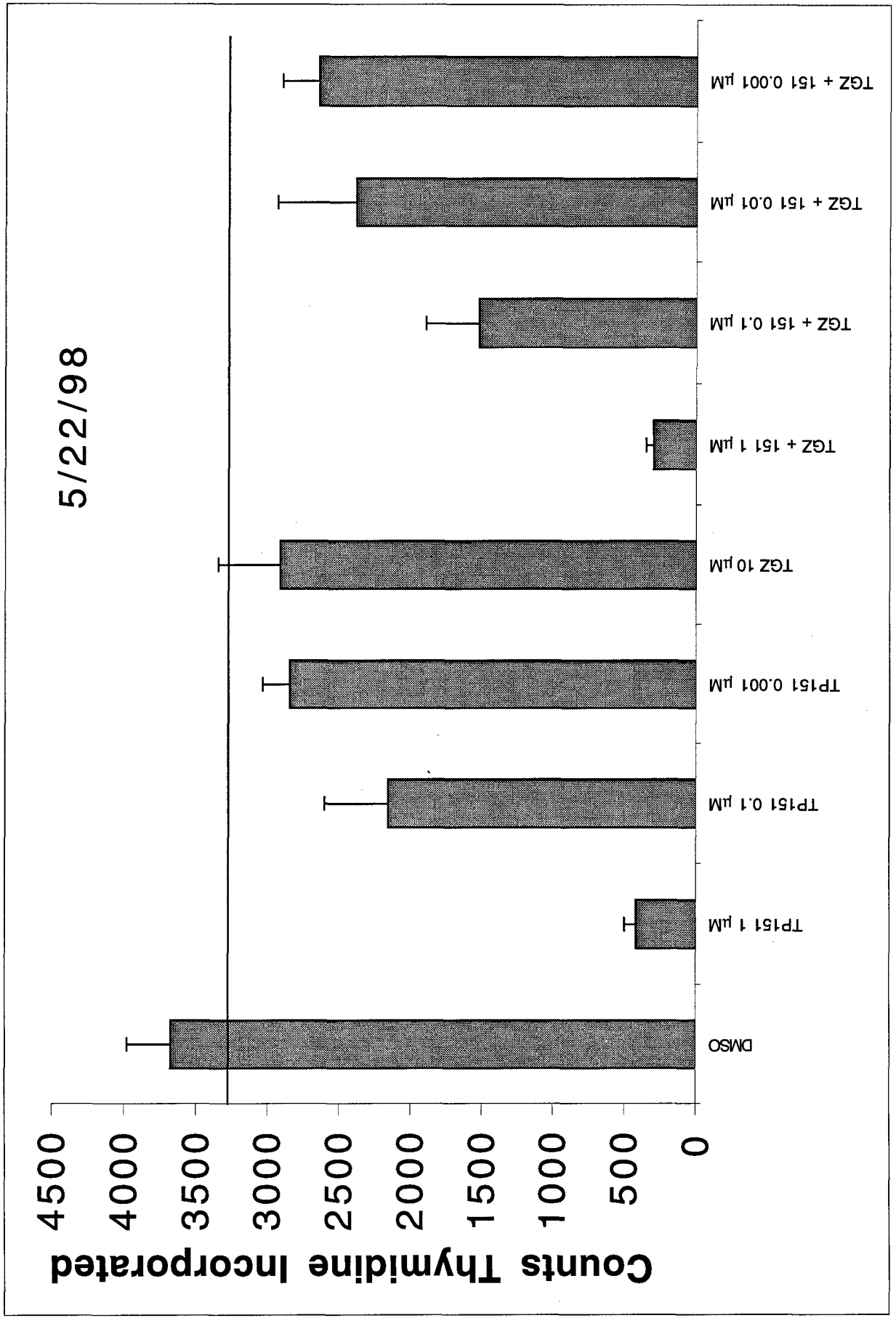


1000 cells per well plated in 10% FBS, DMEM/F12 with phenol red
72 hours treatment with compounds
2 hours thymidine pulse

TP-151 = CDDO
TGZ = Troglitazone

Figure 9

21MT-1 ER Neg Cells: Growth Inhibition by TP-151 and Troglitazone



1000 cells per well plated in 10% FBS growth media
72 hours in compounds
2 hours thymidine pulse

TP-151 = CDDO
TGZ = Troglitazone

Figure 10

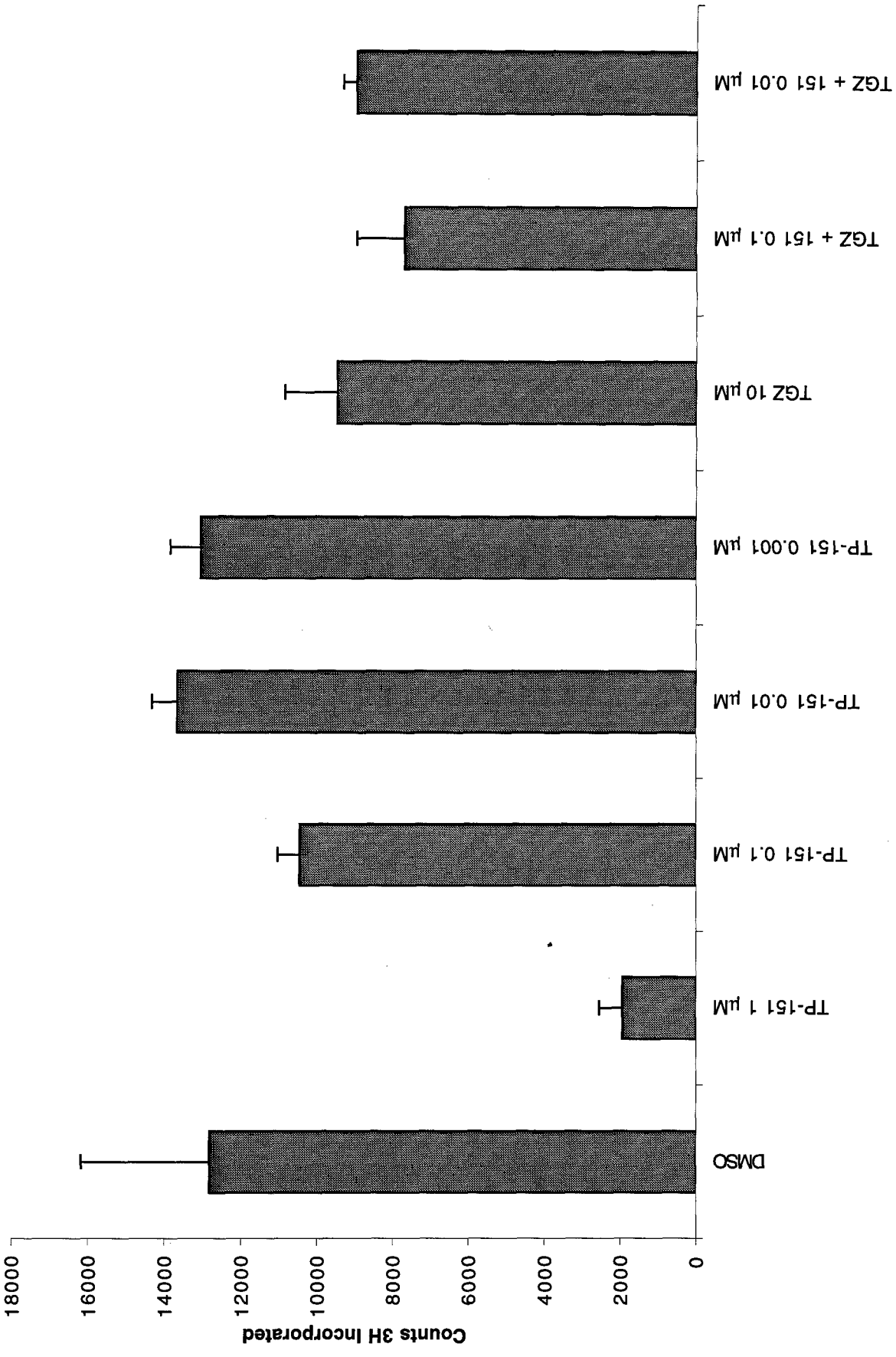
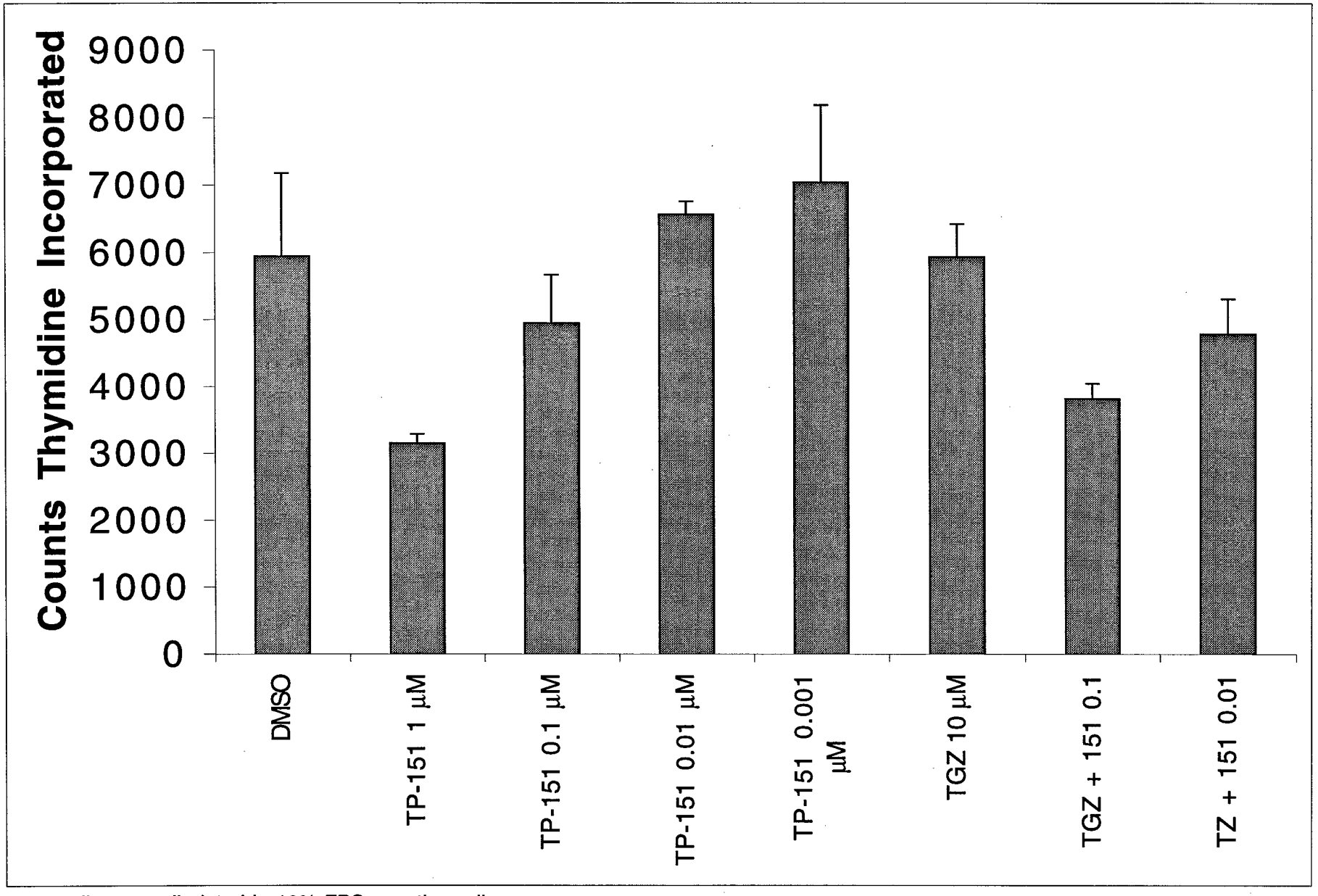

Growth Inhibition of 21-MT-2 ER-Negative Breast Cancer Cells
Counts 3H Incorporated
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
DMSO
TP-151 1 μM
TP-151 0.1 μM
TP-151 0.01 μM
TP-151 0.001 μM
TGZ 10 μM
TGZ + 151 0.1 μM
TGZ + 151 0.01 μM
TP-151 = CDDO
TGZ = Troglitazone

Figure 11

21-NT ER Neg Breast Cancer Cells: Growth Effects by TP-151 and TGZ

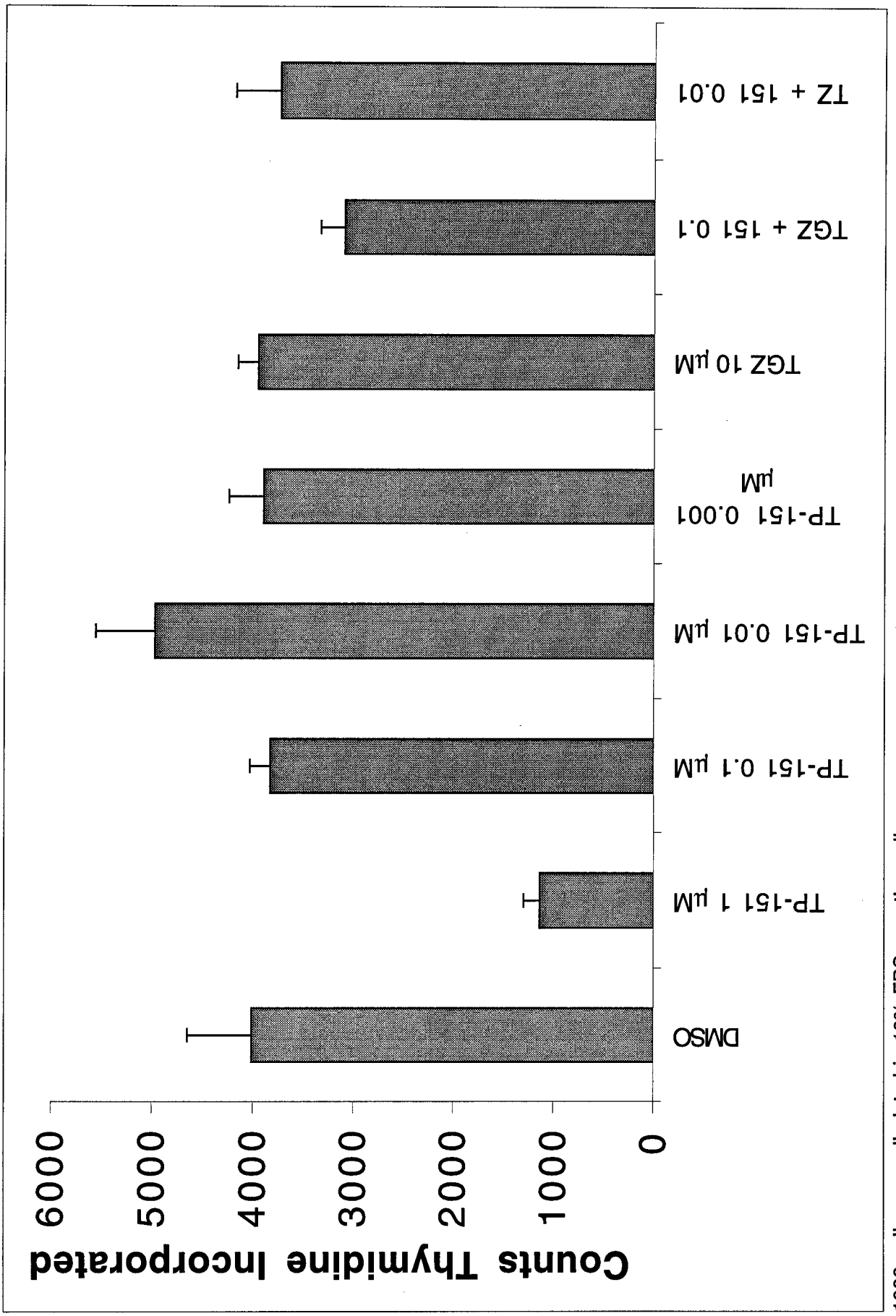


1000 cells per well plated in 10% FBS growth media
72 hours treatment with compounds
2 hours thymidine pulse

TP-151 = CDDO
TGZ = Troglitazone

Figure 12

21-PT ER Neg Breast Cancer Cells: Growth Effects by TP-151 and TGZ

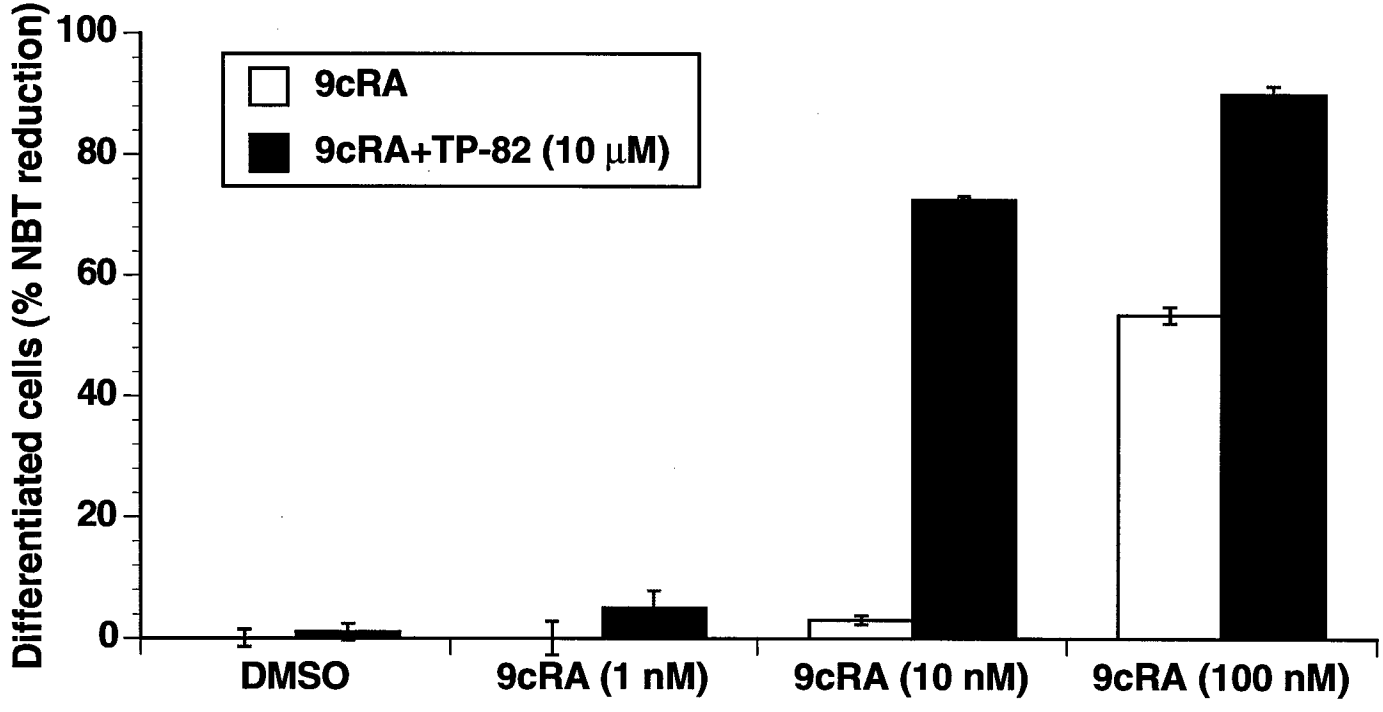


1000 cells per well plated in 10% FBS growth media
72 hours treatment with compounds
2 hours thymidine pulse

TP151 = CDDO
TGZ = Troglitazone

Figure 13

NB4 Human Promyelocytic Leukemia Cell Differentiation with 9-cis-Retinoic Acid and TP-82



NB4 human promyelocytic leukemia were incubated for 4 days with test compounds above. NBT (nitroblue tetrazolium) reduction was used as a differentiation marker.

(7) CONCLUSIONS

We have demonstrated the validity of a new approach to inhibition of carcinogenesis. We have shown that it is possible to synthesize new synthetic triterpenoids that are potent inhibitors of the de novo formation of the enzymes, inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2), that are known to be important etiologic factors in the development of cancer. During the first year of this project, we have published one manuscript on triterpenoid synthesis, which acknowledges support from this grant. During the second year of this project, we have published two more articles which acknowledge support from the grant. These articles show the biological relevance of new triterpenoids and indicate that chemical synthesis can yield highly potent new agents. Further studies on the synthesis and testing of new triterpenoids should now be pursued, with the eventual goal being to find a triterpenoid that could be used for chemoprevention of breast cancer in women at high risk for development of this disease.

(8) REFERENCES

Anzano, M. A., Byers, S.W., Smith, J. M., Peer, C. W., Mullen, L. T., Brown, C. C., Roberts, A. B., and Sporn, M. B. Prevention of breast cancer in the rat with 9-*cis* - retinoic acid as a single agent and in combination with tamoxifen. Can. Res., 54: 4614-4617, 1994.

Honda, T., Finlay, H. J., Gribble, G. W., Suh, N., and Sporn, M. B. New enone derivatives of oleanolic acid and ursolic acid as inhibitors of nitric oxide production in mouse macrophages. Bioorg. Med. Chem. Lett., 7: 1623-1628, 1997.

Hong, W. K., and Sporn, M. B. Recent advances in cancer chemoprevention. Science, in press, 1997.

Huang, M. T., Ho, C. T., Wang, Z. Y., Ferraro, T., Lou, Y. R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J. D., and Conney, A. H. Inhibition of skin tumorigenesis by rosemary and its constituents carnesol and ursolic acid. Cancer Res., 54: 701-708, 1994.

Liu, X-H., and Rose, D.P. Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines. Cancer Res. 56: 5125-5127, 1996.

Marnett, L.J. Aspirin and the potential role of prostaglandins in colon cancer. Cancer Res. 52:5575-5589, 1992.

Nishino, H., Nishino, A., Takayasu, J., Hasegawa, T., Iwashima, A., Hirabayashi, K., Iwata, S., and Shibata, S. Inhibition of the tumor-promoting action of 12-*O*-tetradecanoylphorbol-13-acetate by some oleanane-type triterpenoid compounds. Cancer Res., 48: 5210-5215, 1988.

Ohshima, H., and Bartsch, H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutat. Res., 305: 253-264, 1994.

Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell, 87: 803-809, 1996.

Prescott, S. M., and White, R. L. Self-promotion? Intimate connections between APC and prostaglandin H synthase-2. Cell, 87: 783-786, 1996.

Sheng, H., Shao, J., Kirkland, S.C., Isakson, P., Coffey, R.J., Morrow, J., Beauchamp, R.D., and DuBois, R.N. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J Clin Invest., 99:9:2254-2259, 1997.

Sporn, M. B. The war on cancer. Lancet, 347:1377-81, 1996.

Sporn, M. B., and Roberts, A. B. Peptide growth factors and inflammation, tissue repair, and cancer. J. Clin. Invest., 78: 329-332, 1986.

Takahashi, M., Fukuda, K., Ohata, T., Sugimura, T., and Wakabayashi, K. Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat colon tumors induced by azoxymethane. Cancer Res., 57: 1233-1237, 1997.

Tamir, S., and Tannenbaum, S. R. The role of nitric oxide (NO) in the carcinogenic process. Biochim. Biophys. Acta., 1288: F31-F36, 1996.

Thomsen, L. L., Miles, D. W., Happerfield, L., Bobrow, L. G., Knowles, R. G., and Moncada, S. Nitric oxide synthase activity in human breast cancer. British J. of Cancer 72: 41-44, 1995.

(9) APPENDICIES

1. Honda, T., Finlay, H. J., Gribble, G. W., Suh, N., and Sporn, M. B. New enone derivatives of oleanolic acid and ursolic acid as inhibitors of nitric oxide production in mouse macrophages. Bioorg. Med. Chem. Lett., 7: 1623-1628, 1997.

2. Suh, N., Honda, T., Finlay, H.J., Barchowsky, A., Williams, C., Benoit, N. E., Xie, Q., Nathan, C., Gribble, G.W., and Sporn, M.B. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. Cancer Res. 58: 717-723, 1998.

3. Honda, T., Rounds, B.V., Gribble, G.W., Suh, N., Wang, Y., Sporn, M.B. Design and Synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28oic acid, A Novel and Highly Active Inhibitor of Nitric Oxide Production in Mouse Macrophages. Bioorganic & Medicinal Chemistry Letters in press, (1998).

Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 13, pp. 1623-1628, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00279-5

NEW ENONE DERIVATIVES OF OLEANOLIC ACID AND URSOLIC ACID AS INHIBITORS OF NITRIC OXIDE PRODUCTION IN MOUSE MACROPHAGES

Tadashi Honda,[a] Heather J. Finlay,[a] Gordon W. Gribble,*,[a] Nanjoo Suh,[b] and Michael B. Sporn*,[b]

[a]*Department of Chemistry, Dartmouth College, Hanover, NH 03755, U.S.A. and*
[b]*Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755, U.S.A.*

Abstract: New derivatives of 3-oxoolean-1-en-28-oic acid and 3-oxours-1-en-28-oic acid were synthesized. Nine of them showed significant inhibitory activity against interferon-γ-induced nitric oxide production in mouse macrophages when assayed at the 1 μM level. 3,12-Dioxoolean-1,9-dien-28-oic acid (**3**) had the highest activity (IC_{50}, 0.9 μM). © 1997 Elsevier Science Ltd.

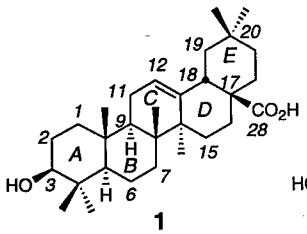
Introduction

Many oleanane and ursane triterpenoids are reported to have interesting biological, pharmacological, or medicinal activities similar to those of retinoids and steroids, such as anti-inflammatory activity, suppression of tumor promotion, suppression of immunoglobulin synthesis, protection of the liver against toxic injury, induction of collagen synthesis, and induction of differentiation in leukemia or teratocarcinoma cells.[1] However, there has never been a systematic study of structure–activity relationships in this set of molecules. Bioassay-directed systematic drug design and synthesis of derivatives of oleanolic acid (**1**) and ursolic acid (**2**), which are commercially available, are of great value in discovering new structures with significant biological activity.

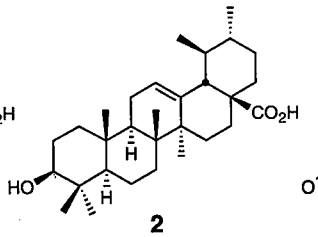
The high output of nitric oxide (NO) produced by inducible nitric oxide synthase (*i*-NOS), which is expressed in activated macrophages, plays an important role in host defense. However, excessive production of NO also can destroy functional normal tissues during acute and chronic inflammation.[2] Thus, inhibitors of NO production in macrophages are potential anti-inflammatory drugs. For this purpose we synthesized oleanolic and ursolic acid derivatives and tested them as inhibitors of NO production. We have found a series of new derivatives of 3-oxoolean-1-en-28-oic acid and 3-oxours-1-en-28-oic acid to have significant inhibitory activity against interferon-γ (IFN-γ)-induced NO production in mouse macrophages.[3] In particular, 3,12-dioxoolean-1,9-dien-28-oic acid (**3**) had the highest activity (IC_{50}, 0.9 μM) in this group of compounds. In this communication, the synthesis, inhibitory activity, and structure–activity relationships are reported for these compounds.

Discovery of Lead Compounds

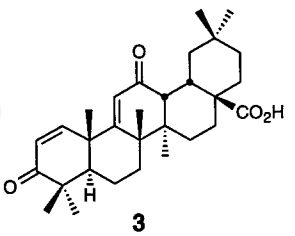
When we started this project, we had no information about a lead compound. Therefore, about sixty oleanolic and ursolic acid derivatives, e.g., 3-hydroxy-, 3-chloro-, 2-chloro-, C-ring cleaved, and 3-oxo-derivatives (including compounds **4**–**7**), were initially randomly synthesized. In the preliminary screen of these



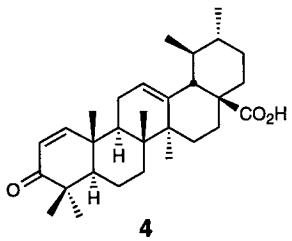
1



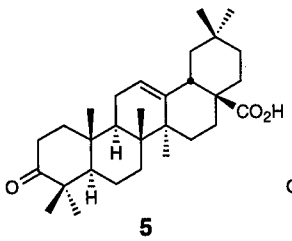
2



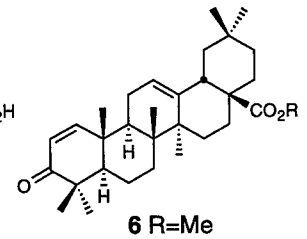
3



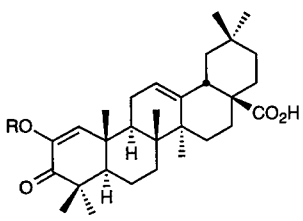
4



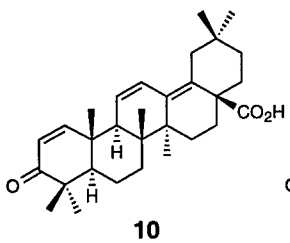
5



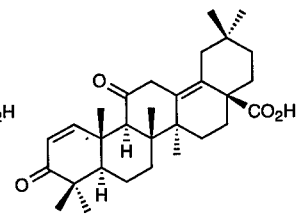
6 R=Me
7 R=H



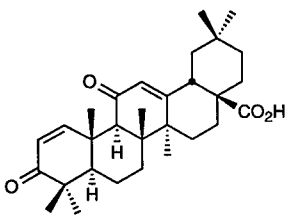
8 R=Me
9 R=H



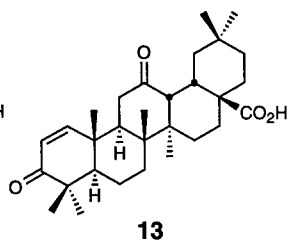
10



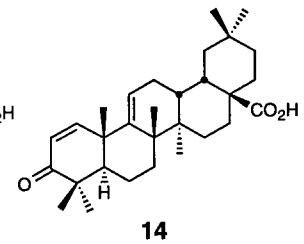
11



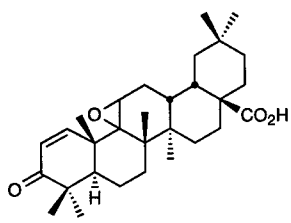
12



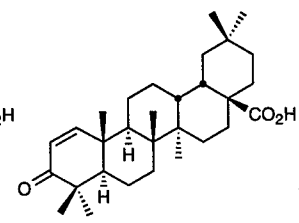
13



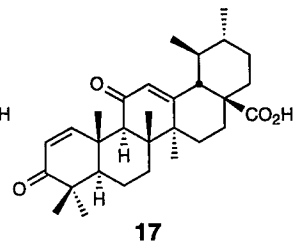
14



15

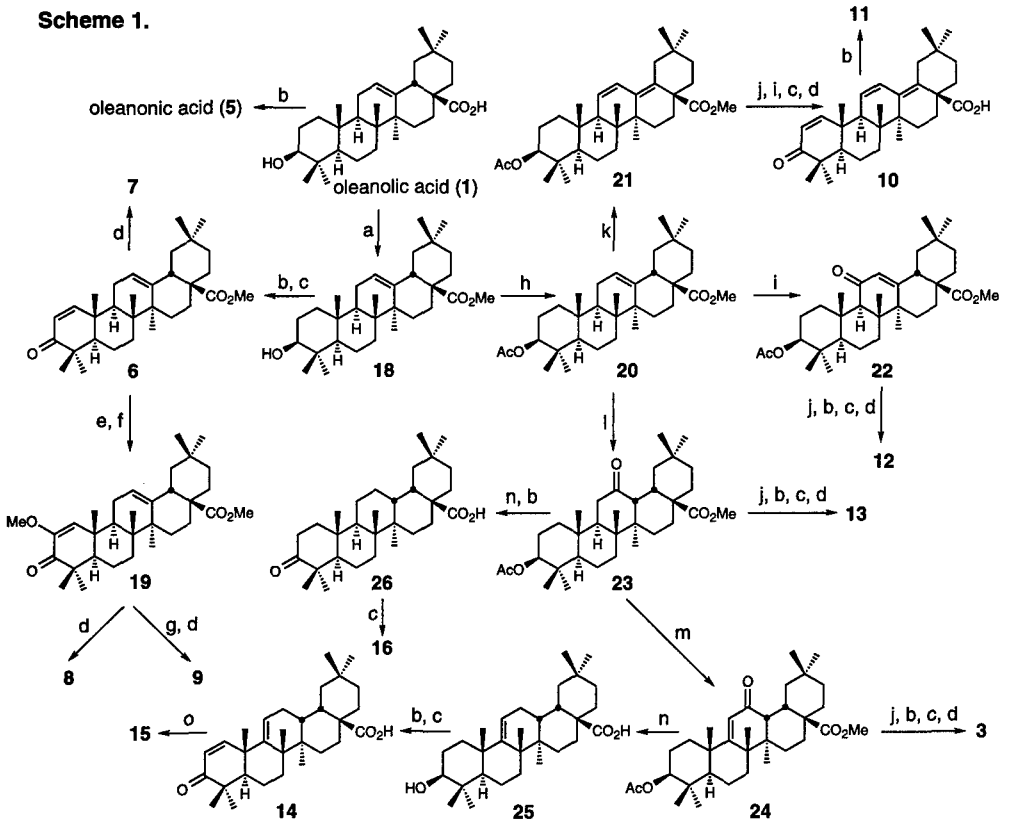


16

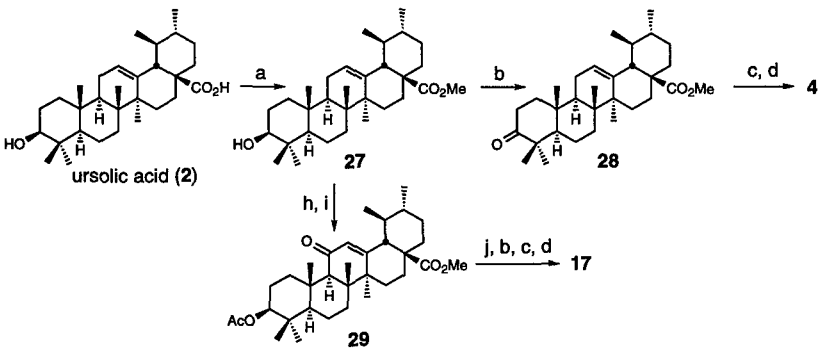


17

Scheme 1.



Scheme 2.



a: CH_2N_2/Et_2O/THF, b: Jones, c: PhSeCl/AcOEt; 30%H_2O_2/THF, d: LiI/DMF, e: 30%H_2O_2/NaOH/THF, f: MeONa, g: HCl/AcOH, h: Ac_2O/pyr., i: CrO_3/pyr./CH_2Cl_2, j: KOH/MeOH, k: SeO_2/AcOH, l: 30%H_2O_2/AcOH, m: Br_2/HBr/AcOH, n: NH_2NH_2/KOH/diethylene glycol, o: *m*-CPBA/CH_2Cl_2

derivatives for inhibition of IFN-γ-induced NO production in mouse macrophages, 3-oxoolean-1,12-dien-28-oic acid (**7**) was found to show significant activity (IC_{50}, 6.0 μM).

Design and Synthesis of New Derivatives

When **7** is compared with the other derivatives (e.g., **1**, **2**, and **4–6**), it has the following features: first, it is an oleanane; second, it has a 1-en-3-one structural unit in ring A; third, it has a carboxyl group at C-17. On the basis of these features of **7**, various derivatives with a 1-en-3-one structural unit in ring A and a carboxyl group at C-17 (**3** and **8–17**) were designed. The synthesis of these newly designed derivatives and compounds **4–7** are illustrated in Schemes 1 and 2.[4] Oleanonic acid (**5**)[5] was prepared in quantitative yield by Jones oxidation of **1**. Enone ester **6** was synthesized by Jones oxidation of methyl oleanolate (**18**)[6] (yield, 90%), followed by introduction of a double bond at C-1 with phenylselenenyl chloride in ethyl acetate and sequential addition of 30% hydrogen peroxide[7] ($PhSeCl\text{-}H_2O_2$) (yield, 70%). Enone **7** was synthesized in 88% yield by halogenolysis of **6** with lithium iodide (LiI) in dimethylformamide (DMF).[8] Enone **8** was synthesized in 35% yield by halogenolysis of ester **19** with LiI in DMF, which was prepared by epoxidation of **6** with alkaline hydrogen peroxide (yield, quantitative), followed by sodium methoxide (yield, quantitative).[9] Diosphenol **9** was synthesized by demethylation of the methyl enol ether at C-2 of **19** with hydrochloric acid in acetic acid (yield, 88%), followed by halogenolysis (yield, 18%). Diene **10** was synthesized by alkaline hydrolysis of acetate **21** (yield, quantitative), which was prepared from methyl acetyloleanolate (**20**)[6] according to a known method,[10] sequential Ratcliffe oxidation[11] (yield, 90%), introduction of a double bond at C-1 (yield, 66%), and halogenolysis (yield, 56%). Deconjugated enone **11** was prepared in 28% yield by Jones oxidation of **10**. Bis-enone **12** was synthesized by alkaline hydrolysis of acetate **22** (yield, quantitative), which was prepared from **20** according to our improvement on a known method,[12] sequential Jones oxidation (yield, 91%), introduction of a double bond at C-1 (yield, 97%), and halogenolysis (yield, 43%).[13] Enone **13** was synthesized in 46% yield from C-12 ketone **23**[14] according to the same synthetic route as for **12**. Bis-enone **3** was also synthesized in 26% yield from enone **24**[15] according to the same synthetic route as for **12**. Enone **14** was synthesized by Jones oxidation of acid **25**[16] (yield, 95%), followed by introduction of a double bond at C-1 (yield, 80%). Epoxide **15**[17] was prepared in 46% yield by epoxidation of **14** with *m*-chloroperbenzoic acid in methylene chloride. Enone **16** was prepared in 51% yield by introduction of a double bond at C-1 of acid **26**[18] with $PhSeCl\text{-}H_2O_2$. Enone **4** was prepared by introduction of a double bond at C-1 of ketone **28**[19] with $PhSeCl\text{-}H_2O_2$ (yield, 66%), followed by halogenolysis (yield, 88%). Bis-enone **17** was synthesized according to the same route as for **12** in 42% yield from enone **29**, which was prepared from **27** according to our improvement on a known method,[16,20]

Biological Results and Discussion

The inhibitory activities [IC_{50} (μM) value] of compounds **1–17** and hydrocortisone (a positive control) on IFN-γ-induced NO production in mouse macrophages are shown in the Table. Nine of the new derivatives of 3-oxoolean-1-en-28-oic acid and 3-oxours-1-en-28-oic acid showed significant activity at the 1 μM level. Six of them were superior to the lead compound **7**. Modification of the A and C ring affected activity strongly. In particular, bis-enone type compounds **3** and **12** showed high activity. Surprisingly, ursolic acid (**2**) stimulated NO production although ursolic acid derivatives **4** and **17** showed inhibitory activity. None of the synthesized derivatives were toxic to primary mouse macrophages at 40 μM.

These preliminary results revealed some interesting structure–activity relationships as follows:

(1) In the A ring, a 1-en-3-one structural unit without a substituent is important for significant activity. For example, 1-en-3-one **7** is much more active in comparison with diosphenol **9**, diosphenol methyl ether **8**, C-3 ketone **5**, and C-3 alcohol **1**.

(2) In the C ring: (a) a carbonyl group at C-11 and/or C-12 is important; (b) particularly, an insertion of a double bond at the α position of C-11 and/or C-12 ketone enhances the activity. Bis-enone **3** with 1-en-3-one and 9-en-12-one structural units showed the highest activity. Bis-enone **12**, C-11 ketone **11**, and C-12 ketone **13** also showed high activity, and were more active than **7**. Bis-enone **17** which has an ursane skeleton is also more active than **4**.

(3) At C-17, a carboxyl group (e.g., **7**) gives much more activity than a methoxycarbonyl group (e.g., **6**). Hydrophilic groups seem to be much better than hydrophobic groups.

(4) The oleanane skeleton is more active than the ursane skeleton. **7** and **12** are more active than **4** and **17**, respectively.

On the basis of these structure–activity relationships, further lead optimization is in progress. Studies on the mode of action of these derivatives also are in progress.

Table. IC_{50} (μM) [a] Values for Inhibition of IFN-γ-Induced NO Production in Mouse Macrophages [3]

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
hydrocortisone	0.015	**10**	9.7
3	0.9	**4**	17.6
12	1.8	**9**	26.5
11	2.6	**8**	30.0
13	3.3	**15**	35.5
17	5.1	**5**	37.1
14	5.2	**6**	40.0
7	6.0	oleanolic acid (**1**)	40.0
16	8.5	ursolic acid (**2**)	stimulation [b]

a: All IC_{50} (μM) values were determined over the range of 0.1–40 μM for each compound, except for hydrocortisone, using the computer calculation program Tablecurve® (all were fitted to a log–dose response curve.) Values are an average of two separate experiments.

b: Ursolic acid (**2**) is strongly toxic to primary mouse macrophages (toxic above 5–10 μM).

Acknowledgments

We thank Drs. Carl Nathan and Qiao-wen Xie for expert advice on the preparation of macrophages and the nitric oxide assay. This investigation was supported by funds from the Norris Cotton Cancer Center and U.S. Dept. of Defense Grant # DAMD17-96-1-6163. M.B.S. is the Oscar M. Cohn Professor. Mass spectral data were kindly furnished by Drs. Tim Barden, Mark G. Saulnier, and Stephen Wright.

References and Notes

1. (a) Tang, W.; Elsenbrand, G. In *Chinese Drugs of Plant Origin*; Springer-Verlag: Berlin, 1992. (b) Yu, L.; Ma, R.; Wang, Y.; Nishino, H.; Takayasu, J.; He, W.; Chang, M.; Zhen, J.; Liu, W.; Fan, S. *Int. J. Cancer* **1992**, *50*, 635. (c) Umehara, K.; Takagi, R.; Kuroyanagi, M.; Ueno, A.; Taki, T.; Chen, Y-J. *Chem. Pharm. Bull.* **1992**, *40*, 401. (d) Huang, M-T.; Ho, C-T.; Wang, Z. Y.; Ferrao, T.; Lou, Y-R.; Stauber, K.; Ma, W.; Georgiadis, C.; Laskin, J. D.; Conney, A. H. *Cancer Res.* **1994**, *54*, 701. (e) Liu, J.; Liu, Y.; Mao, Q.; Klaassen, C. D. *Fundam. Appl. Toxicol.* **1994**, *22*, 34.
2. (a) Knowles, R. G.; Moncada, S. *Biochem. J.* **1994**, *298*, 249. (b) Nathan, C. F.; Xie, Q. *J. Biol. Chem.* **1994**, *269*, 13725. (c) Anggard, E. *Lancet* **1994**, *343*, 1199.
3. Briefly, the procedure for this assay is as follows: Macrophages were harvested from female mice injected intraperitoneally four days previously with 4% thioglycollate. These cells were seeded in 96-well tissue culture plates and incubated with 4 ng/mL IFN-γ in the presence or absence of inhibitory test compounds. After 48 hours NO production (measured as nitrite by the Griess reaction) was determined. Full details of the assay are given in reference 21.
4. All new compounds **3**, **4**, and **6–17** exhibited satisfactory spectral data including high-resolution mass spectra.
5. Simonsen, J.; Ross, W. C. J. In *the Terpenes*; Cambridge University: Cambridge, 1957; Vol 5, pp 221–285.
6. Frazier, D.; Noller, C. R. *J. Am. Chem. Soc.* **1944**, *66*, 1267.
7. Sharpless, K. B.; Lauer, R. F.; Teranishi, A. Y. *J. Am. Chem. Soc.* **1973**, *95*, 6137.
8. Dean, P. D. G. *J. Chem. Soc.* **1965**, 6655.
9. Kurata, Y.; Hirota, H.; Honda, T.; Takahashi, T. *Chem. Pharm. Bull.* **1987**, *35*, 837.
10. Ruzicka, L.; Grob, A.; van der Sluys-Veer, F. C. *Helv. Chim. Acta.* **1939**, *22*, 788.
11. Ratcliffe, R.; Rodehorst, R. *J. Org. Chem.* **1970**, *35*, 4000.
12. Mower, N.; Green, J.; Spring, F. S. *J. Chem. Soc.* **1944**, 256.
13. Deconjugated enone **11** was also produced in 22% yield by halogenolysis with LiI in DMF.
14. Ruzicka, L.; Cohen, S. L. *Helv. Chim. Acta.* **1937**, *20*, 804.
15. Picard, C. W.; Sharples, K. S.; Spring, F. S. *J. Chem. Soc.* **1939**, 1045.
16. Barton, D. H. R.; Holness, N. J. *J. Chem. Soc.* **1952**, 78.
17. Epoxide **15** is thought to be the β-epoxide by the $W_{1/2}$ value of the C-11 proton [δ, 3.05 ppm; $W_{1/2}$= 3.4 Hz ($CDCl_3$)] in ^1H NMR, although its structure has not been confirmed.
18. Dietrich, P.; Jeger, O. *Helv. Chim. Acta.* **1950**, *33*, 711.
19. Jacobs W. A.; Fleck, E. E. *J. Biol. Chem.* **1931**, *92*, 487.
20. Ewen, E. S.; Spring F. S. *J. Chem. Soc.* **1943**, 523.
21. (a) Ding, A.; Nathan, C. F.; Graycar, J.; Derynck, R.; Stuehr, D. J.; Srimal, S. *J. Immunol.* **1990**, *145*, 940. (b) Bogdan, C.; Paik, J.; Vodovotz, Y.; Nathan, C. F. *J. Biol. Chem.* **1992**, *267*, 23301.

(*Received in USA* 24 *March* 1997; *accepted* 20 *May* 1997)

[CANCER RESEARCH 58, 717–723, February 15, 1998]

Novel Triterpenoids Suppress Inducible Nitric Oxide Synthase (iNOS) and Inducible Cyclooxygenase (COX-2) in Mouse Macrophages[1]

Nanjoo Suh, Tadashi Honda, Heather J. Finlay, Aaron Barchowsky, Charlotte Williams, Nicole E. Benoit, Qiao-wen Xie, Carl Nathan, Gordon W. Gribble, and Michael B. Sporn[2]

Department of Pharmacology and Norris Cotton Cancer Center, Dartmouth Medical School [N. S., A. B., C. W., N. E. B., M. B. S.] and Department of Chemistry, Dartmouth College, Hanover, New Hampshire 03755 [T. H., H. J. F., G. W. G.], and Department of Medicine, Cornell University Medical College, New York, New York 10021 [Q-w. X., C. N.]

ABSTRACT

We have synthesized more than 80 novel triterpenoids, all derivatives of oleanolic and ursolic acid, as potential anti-inflammatory and chemopreventive agents. These triterpenoids have been tested for their ability to suppress the *de novo* formation of two enzymes, inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2), using IFN-γ-stimulated primary mouse macrophages or lipopolysaccharide (LPS)-activated RAW 264.7 macrophages as assay systems. Two synthetic oleananes, 3,12-dioxoolean-1-en-28-oic acid (TP-69) and 3,11-dioxoolean-1,12-dien-28-oic acid (TP-72), were highly active inhibitors of *de novo* formation of both iNOS and COX-2. Both TP-69 and TP-72 blocked the increase in iNOS or COX-2 mRNA induced by IFN-γ or LPS. In addition, TP-72 suppressed NF-κB activation in primary macrophages treated with the combination of IFN-γ and LPS or IFN-γ and tumor necrosis factor. The 3-α(axial)-epimer of ursolic acid suppressed *de novo* formation of COX-2, in contrast to naturally occurring 3-β(equatorial)-ursolic acid. Inhibitory effects of TP-69 or TP-72 on iNOS formation were not blocked by the glucocorticoid receptor antagonist RU-486, indicating that these triterpenoids do not act through the glucocorticoid receptor, nor does TP-72 act as an iNOS or COX-2 enzyme inhibitor when added to RAW cells in which synthesis of these two enzymes in response to LPS has already been induced. It may be possible to develop triterpenoids as useful agents for chemoprevention of cancer or other chronic diseases with an inflammatory component.

INTRODUCTION

One of the major needs in cancer prevention is the development of effective and safe new agents for chemoprevention. In particular, there is a unique need for chemopreventive agents targeted at mechanisms known to be involved in the process of carcinogenesis (1). In recent years, there has been a resurgence of interest in the study of mechanisms of inflammation that relate to carcinogenesis and in the use of such mechanisms as the basis for development of new chemopreventive agents.

The concept that inflammation and carcinogenesis are related phenomena has been the subject of many previous studies that have attempted to link these two processes in a mechanistic fashion (2–4). The enzymes that mediate the constitutive synthesis of NO and prostaglandins from arginine and arachidonate, respectively, have relatively little significance for either inflammation or carcinogenesis. In contrast, iNOS[3] (EC 1.14.13.39) and inducible cyclooxygenase (COX-2; EC 1.14.99.1) have critical roles in the response of tissues to injury or infectious agents. These inducible enzymes are essential components of the inflammatory response, the ultimate repair of injury, and carcinogenesis (5–10). Although physiological activity of iNOS and COX-2 may provide a definite benefit to the organism, aberrant or excessive expression of either iNOS or COX-2 has been implicated in the pathogenesis of many disease processes, as diverse as septic shock, cardiomyopathy, acute and chronic neurodegenerative disease, rheumatoid arthritis, and carcinogenesis (11–19).

Immense effort has been devoted to developing new molecules that are direct inhibitors of the enzymatic activity of either iNOS or COX-2. However, an alternative approach is to find new agents that can prevent expression of the respective genes coding for these enzymes. Glucocorticoids and TGF-β are such molecules; they both suppress transcription or translation of *iNOS* and *COX-2* genes (20–25). A rationale thus exists to develop more selective agents for suppression of genes that might be overexpressed during the inflammatory or carcinogenic process. In this report, we attempt to apply this strategy for the development and evaluation of new triterpenoids.

Although triterpenoids are widely used for medicinal purposes in many Asian countries, this class of molecules, which resemble steroids in their chemical structure, biogenesis, and pleiotropic actions, has not impacted on the practice of Western medicine. Triterpenoids, like the steroids, are formed in nature by the cyclization of squalene, with the retention of all 30 carbon atoms in molecules such as OA and UA (Fig. 1). Although OA and UA are known to have numerous pharmacological activities, the potency of these naturally occurring molecules is relatively weak. Chemical synthesis of new steroid analogues has provided many useful derivatives that are more potent and specific than natural parent structures. With this as a model and considering the known anti-inflammatory and anticarcinogenic activities of OA and UA (26–29), we have synthesized (30) and characterized a new series of synthetic triterpenoid analogues as potential inhibitors of inflammation and carcinogenesis using suppression of the formation of nitric oxide and prostaglandins as assay systems. We report here the structures and activities of three of the most potent of these derivatives of OA and UA. These new agents are significantly more active than their parent molecules.

MATERIALS AND METHODS

Reagents

Details of the synthesis of TP-69 and TP-72 (see Fig. 1 for structures) have been published (30). TP-52 (3-α-OH UA) was synthesized by Jones oxidation of UA, followed by Meerwein-Ponndorf reduction. Recombinant mouse IFN-γ (LPS content, <10 pg/ml) was purchased from Genzyme (Cambridge, MA); NF-κB oligonucleotide was purchased from Promega Corp. (Madison, WI); goat polyclonal COX-1, COX-2 IgG, and anti-goat IgG peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); TGF-β_1 was from R&D (Minneapolis, MN); and enzyme immunoassay reagents for PGE_2 assays were from Cayman Co. (Ann Arbor, MI). TNF-α was provided by Dr. Jan Vilcek (New York University Medical Center, New York, NY). LPS (from *Escherichia coli* 0111:B4 γ-irradiated) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Received 10/14/97; accepted 12/19/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This research was supported by Norris Cotton Cancer Center Grant CA-23108, NIH Grant R01 AI 34543, the Oliver S. and Jennie R. Donaldson Charitable Trust, and DOD/AMRD Award 17-96-1-6163.

[2] To whom requests for reprints should be addressed, at Department of Pharmacology and Toxicology, Dartmouth Medical School, 7650 Remsen, Hanover, NH 03755. Phone: (603) 650-6557; Fax: (603) 650-1129; E-mail: Michael.Sporn@dartmouth.edu.

[3] The abbreviations used are: iNOS, inducible nitric oxide synthase; TGF, transforming growth factor; COX, cyclooxygenase; OA, oleanolic acid; UA, ursolic acid; PG, prostaglandin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; NF, nuclear factor.

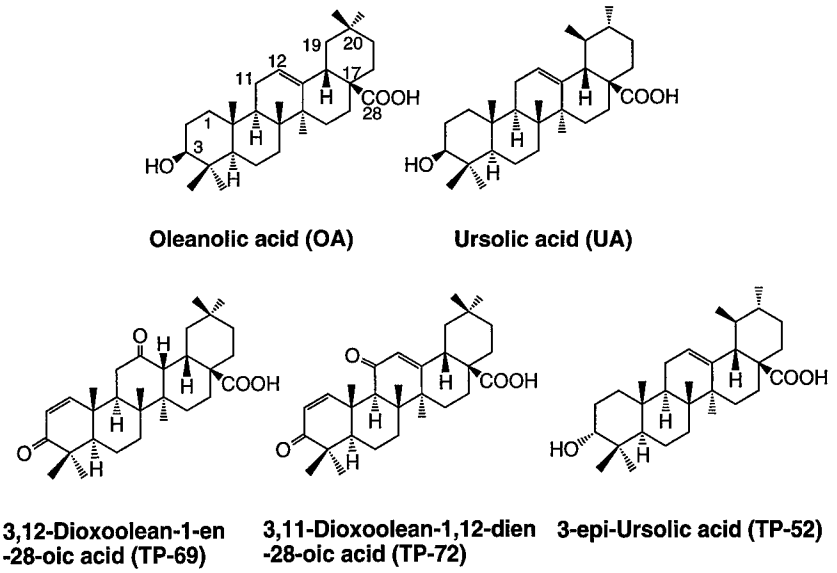


Fig. 1. Chemical structures of the natural triterpenoids, OA and UA, and the synthetic analogues, 3,12-dioxoolean-1-en-28-oic acid (TP-69), 3,11-dioxoolean-1,12-dien-28-oic acid (TP-72), and 3-epi-ursolic acid (TP-52).

Triterpenoids were dissolved in DMSO before addition to cell cultures or enzyme assays; final concentrations of DMSO were 0.1% or less. Controls with DMSO alone were run in all cases.

Cell Culture

RAW 264.7 cells were maintained in RPMI 1640 with 10% fetal bovine serum. These cells were treated with LPS (10 ng/ml) for 6–18 h to induce iNOS or COX-2. To obtain primary macrophages, female CD-1 mice, 5–10 weeks of age (Charles River Breeding Laboratories, Wilmington, MA), were injected i.p. with 2 ml of 4% thioglycollate broth (Difco Laboratories, Detroit, MI). Four days after injection, peritoneal macrophages were harvested and processed as described (31). Cells were seeded in 96-well plates at 2×10^5 cells/well and stimulated with IFN-γ. Triterpenoids were added at the same time.

Measurement of iNOS Enzyme Activity, Protein, and mRNA Levels

NO Production in Mouse Macrophages and RAW 264.7 Cell Line. Nitrite accumulation was used as an indicator of NO production in the medium and was assayed by the Griess reaction (32). One hundred μl of Griess reagent were added to 100 μl of each supernatant from LPS, IFN-γ, or triterpenoid-treated cells in triplicate. The protein determination was performed by Bradford protein assay. The plates were read at 550 nm against a standard curve of sodium nitrite.

Inhibition of LPS-induced iNOS Enzyme Activity. For the assay in intact cells, RAW 264.7 cells were plated in 100-mm tissue culture dishes (4×10^6 cells) and incubated for 12 h with LPS. The cells were washed twice with PBS. Cells were harvested and plated into a 96-well plate (2×10^5 cells/well) and incubated in the absence or presence of test compounds for 12 h further, with no LPS in the medium. The supernatants were removed, and the Griess assay was performed as above. For the assay in cell lysates, RAW 264.7 cells were washed three times with PBS, scraped into cold PBS, and centrifuged at 500 × *g* for 10 min at 4°C. The cell pellet was resuspended in 0.5 ml 40 mM Tris-buffer (pH 8.0) containing 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 μg/ml aprotinin, and 100 μM phenylmethylsulfonyl fluoride and lysed by three freeze-thaw cycles. Aliquots of the lysate were used for Bradford protein assay. iNOS enzyme activity was measured as described (21). Briefly, 10–20 μg of cell lysate protein were incubated in 20 mM Tris-HCl (pH 7.9), containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM DTT, and 2 mM each of L-arginine and NADPH. The reaction was carried out in duplicate for 180 min at 37°C in 96-well plates. Residual NADPH was oxidized enzymatically as described previously, and the Griess assay was performed as above.

SDS-PAGE and Western Blot Analyses of iNOS Protein in Mouse Macrophages and RAW 264.7 Cells. Mouse macrophages or RAW 264.7 cells were plated in six-well plates (4×10^6 cells/well) and treated, respectively, with IFN-γ or LPS for 18 h. Cells were washed and scraped into cold PBS and then centrifuged at 500 × *g* for 10 min at 4°C. The cell pellets were resuspended in 50 mM Tris-buffer (pH 7.4), 100 mM NaCl, containing 0.5% NP40, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride, and then centrifuged to obtain whole-cell lysates. The proteins (50 μg) were electrophoresed on 7.5% (or 12% in some experiments) reducing SDS-PAGE and transferred in 20% methanol, 25 mM Tris, and 192 mM glycine (pH 8.3) to 0.2-μm (pore size) nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline [25 mM Tris (pH 7.5), 150 mM NaCl, and 0.02% NaN_3) with 0.2% Tween 20 (Tween-TBS) for 1 h, then incubated with rabbit anti-mouse iNOS antiserum (33) for 2–3 h, washed, and finally incubated for 45 min with a 1:10,000 dilution of secondary antibody conjugated with horseradish peroxidase. The membranes were washed and then developed using a chemiluminescence system (enhanced chemiluminescence detection reagents; Amersham).

Northern Blot Analyses of iNOS. RNA was isolated from mouse macrophages and RAW cells by a rapid guanidinium isothiocyanate method (34). The total RNA was denatured in formamide (50%)/formaldehyde (6.5%) sample buffer (65°C for 15 min), run on formaldehyde (1.8%)/agarose (0.8%) gels, and transferred to Nytran nylon membranes (Schleicher & Schuell, Keene, NH). After UV cross-linking, the membranes were prehybridized, hybridized, washed, and exposed to Kodak XAR films. The probe specific for murine iNOS was an 814-bp *Eco*RI/*Acc*I fragment of iNOS cDNA clone B2 (33), which was random primer labeled with 50 μCi of [α-^{32}P]dCTP (6000 Ci/mmol). To control for equal loading of RNA, the membranes were hybridized with a random primer-radiolabeled mouse glyceraldehyde-3-phosphate dehydrogenase cDNA.

Measurement of COX-2 Enzyme Activity, Protein, and mRNA Levels

PGE_2 Production. RAW 264.7 cells were plated in six-well plates and incubated with compounds for 6 h, and then the supernatant culture medium was collected to determine the amount of PGE_2 (Cayman Enzyme Immunoassay kit).

Inhibition of LPS-induced COX-2 Enzyme Activity. RAW 264.7 cells were plated at 1×10^5 cells/well in a 12-well plate and incubated for 6 h with LPS. The cell supernatants were removed, and cells in each well were washed twice with fresh culture medium and allowed to equilibrate in the absence or presence of test compounds for 30 min. The cells were further incubated with 100 μM arachidonic acid for 15 min, with no LPS in the medium. The supernatants were removed and assayed for PGE_2 (35).

Western Blot Analyses of COX-2 in RAW 264.7 Cells. RAW 264.7 cells were treated with LPS for 6 or 18 h. The first steps of the procedure were as described above for Western blots of iNOS. Goat polyclonal COX-1 and COX-2 were used as primary antibodies, and the bands were detected with an

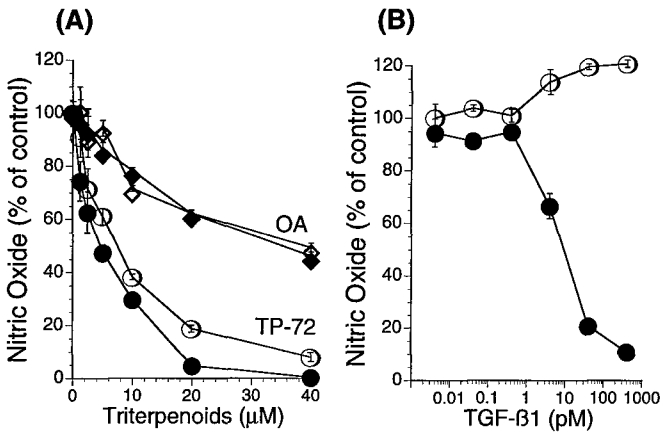


Fig. 2. Inhibitory effects of OA, TP-72, and TGF-β_1 on NO production in primary mouse macrophages (*closed symbols*) and RAW 264.7 cells (*open symbols*). IFN-γ (4 ng/ml) and LPS (10 ng/ml) were used as inducing agents in primary macrophages and RAW 264.7 cells, respectively. Cells were treated for 18 h (LPS) or 48 h (IFN-γ) with inducers and triterpenoids (or TGF-β_1), and NO was then determined in the supernatants by the Griess reaction. NO production in primary macrophages that received no triterpenoid was 4.3 nmol/2 × 10^5 cells; in RAW cells in this figure, NO production was 3.2 nmol/2 × 10^5 cells. Data shown are representative of three typical experiments.

Table 1 *Effect of added triterpenoid or dexamethasone after LPS induction of iNOS enzyme in RAW 264.7 cells*

LPS pretreatment of cells[a]	Addition to LPS-treated RAW cells	NO (nmol/2 × 10^5 cells)[b]
None	DMSO control	0.0 ± 0.0
LPS (10 ng/ml), 12 h	DMSO control	3.9 ± 0.5
	N-Methyl arginine (40 μM)	1.4 ± 0.3[c]
	Dexamethasone (1 μM)	3.6 ± 0.6
	TP-72 (40 μM)	3.9 ± 0.8

[a] RAW 264.7 cells were stimulated for 12 h with LPS (10 ng/ml) in a tissue culture dish, and cells were washed twice with PBS to remove LPS. RAW cells were scraped and placed in a 96-well plate, and *N*-methyl arginine, TP-72, or dexamethasone was then added and incubated for 12 h.

[b] The amount of NO accumulated in the supernatant was detected by Griess assay as described in "Materials and Methods." Data are means ± SE of six samples from two independent experiments. In each experiment, triplicate determinations were made for each treatment.

[c] $P < 0.05$, significantly different from LPS alone, analyzed by Student's *t* test.

anti-goat IgG horseradish peroxidase-conjugated secondary antibody (1:2000 dilution), followed by chemiluminescent detection.

Northern Blot Analysis of COX-2. All procedures were as described above for iNOS. For COX-1 and COX-2 cDNA probes (gifts from Dr. Harvey Herschman, UCLA Cancer Center, Los Angeles, CA), a 2.76-kb insert of COX-1 in the pBluescript vector was digested with *Bsp*HI, and a 2.3-kb insert of COX-2 in the pGEM7 vector was double digested with *Bam*HI and *Xho*I.

Electrophoretic Mobility Shift Assays for NF-κB

NF-κB binding activity in nuclei isolated from uninduced and induced cells was determined by electrophoretic mobility shift assays using methods described previously (36). Nuclear proteins were extracted from macrophages by detergent lysis. Oligonucleotide probes were 5′-end radiolabeled with T4 polynucleotide kinase in the presence of 50 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) to a specific activity of >2 × 10^8 cpm/mg. Five μg of nuclear protein were incubated with 10 fmol of ^{32}P-labeled, double-stranded oligonucleotide containing the NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) binding motif. The specificity of binding was determined by the addition of excess (1.75 pmol) of the same unlabeled oligonucleotide.

RESULTS

Triterpenoids Inhibit NO Production in Mouse Macrophages and RAW 264.7 Cells. Active synthetic triterpenoids caused a dose-dependent inhibition of NO production both in mouse primary macrophages, induced with IFN-γ, and in RAW 264.7 cells, induced with LPS. This inhibition of NO production was not due to toxicity, as determined by trypan blue exclusion and adherent cell protein determination. Triterpenoids TP-69 and TP-72 (Fig. 1) were selected from a primary screening of more than 80 derivatives of OA and UA as the most active ones in the suppression of NO production. TP-69 and TP-72 are synthetic enone analogues of OA that have A- and C-ring modifications with one or two enone functional groups. As shown in the dose-response curves in Fig. 2*A*, IC_{50} values for TP-72 are 3.9 and 6.7 μM in primary macrophages and RAW 264.7 cells, respectively. The data indicate that TP-72 is markedly more active than its parent molecule, OA. TP-69 also inhibited NO production in primary macrophages (IC_{50}, 4.2 μM) and RAW 264.7 cells (IC_{50}, 7.8 μM; curves not shown). Although TGF-β_1 is the most potent known inhibitor of inducible NO formation in primary macrophages (50% inhibition at 8 pM; Refs. 21 and 32), it did not suppress NO formation in transformed RAW 264.7 cells induced with LPS (Fig. 2*B*). In contrast, triterpenoids inhibited NO production in both transformed RAW cells and primary macrophages, suggesting that the inhibition by triterpenoids in RAW cells is not mediated by the action of TGF-β.

TP-72 Does Not Inhibit Intrinsic iNOS Enzyme Activity in RAW 264.7 Cells. We wished to determine whether the inhibitory effect of a triterpenoid on inducible NO production is a direct effect on the intrinsic enzyme activity of iNOS, or whether this inhibition is mediated by some other mechanism. Dexamethasone is known to inhibit *iNOS* gene transcription (37, 38). Table 1 shows that the addition of either TP-72 or dexamethasone to RAW 264.7 cells, which had been pretreated with LPS to induce NOS, does not affect iNOS enzyme activity in the intact cell. In contrast, *N*-methyl arginine, an enzyme substrate analogue, inhibited this enzyme activity (62% inhibition of NO accumulation at 40 μM). Further confirmation of this lack of direct enzyme inhibition by TP-72 was obtained in experiments in which TP-72 was added to lysates of RAW cells that had been pretreated with LPS to induce NOS (Table 2). In these experiments, we performed an enzyme assay for iNOS on the lysates, using arginine as added substrate. There was no inhibition of NO formation (measured as nitrite in the Griess reaction) by TP-72 (20 μM). Thus, assays performed both on intact cells and in cell lysates indicate that TP-72 inhibits NO formation by a mechanism other than direct enzyme inhibition.

Triterpenoids Decrease iNOS mRNA and Protein Levels in Mouse Macrophages and RAW 264.7 Cells. We next investigated whether TP-72 and the related enone, TP-69, might affect levels of iNOS mRNA and the resultant iNOS protein. The data in Fig. 3 show that LPS induces the 4.0-kb iNOS mRNA transcript in RAW cells in a dose-dependent manner, over a range from 1–1000 ng/ml. There is a similar dose-response for the induction of the M_r 130,000 iNOS protein in these same cells. Both the mRNA and protein responses to LPS are markedly attenuated by TP-72 (10 μM). Densitometer scans of the respective blots show approximately 50% inhibition of iNOS protein expression at 1 μg/ml of LPS stimulation. As shown in Fig.

Table 2 *Direct enzyme assay for iNOS on RAW cell lysates*

Pretreatment of cells before lysis	Addition to lysate	iNOS specific activity: NO formed (pmol/mg protein/min)[a]
None	DMSO control	8.0 ± 3.9
LPS (100 ng/ml), 18 h	DMSO control	88.5 ± 17.9
	TP-72 (20 μM)	86.0 ± 10.2
	Dexamethasone (0.2 μM)	83.5 ± 15.2

[a] Average ± SE values were obtained from three separate experiments. Preparation of lysates and iNOS enzyme assay are described in "Materials and Methods." Either TP-72 or dexamethasone was added to lysates from LPS-stimulated RAW 264.7 cells, and iNOS activity was measured.

4*A*, iNOS protein expression is inhibited by TP-69 or dexamethasone after primary mouse macrophages are stimulated by IFN-γ alone (20 ng/ml) or by IFN-γ in combination with TNF-α (100 ng/ml). In contrast to the strong inhibitory effect of the synthetic triterpenoid, TP-69, the naturally occurring parent molecule, OA, was inactive in this assay at an equimolar concentration. Fig. 4*B* shows that IFN-γ is a strong inducer of iNOS mRNA expression in primary mouse macrophages. TP-69 (30 μM) almost totally blocks this induction, with some inhibition of iNOS mRNA levels seen at concentrations as low as 1 μM. In addition, we examined whether these active triterpenoids might suppress constitutive nitric oxide synthase in endothelial cells. In contrast, neither TP-69 nor TP-72 (each at 10 μM) diminished the level of the constitutive NOS in human endothelial cells, as determined by Western blot analysis (data not shown).

Triterpenoids Do Not Act through a Glucocorticoid Receptor-mediated Mechanism. The glucocorticoid antagonist, RU486, was used to determine whether the inhibitory effects of triterpenoids on NO production were mediated through their interaction with the glucocorticoid receptor. Fig. 5 shows that, as expected, inhibitory effects of dexamethasone were reversed by the addition of glucocorticoid receptor antagonist RU486 (1 μM). In contrast, the inhibitory activity of TP-69 and TP-72 on nitric oxide production could not be reversed by RU486. These data strongly suggest that the actions of triterpenoids on the iNOS system are not mediated by their interaction with the glucocorticoid receptor.

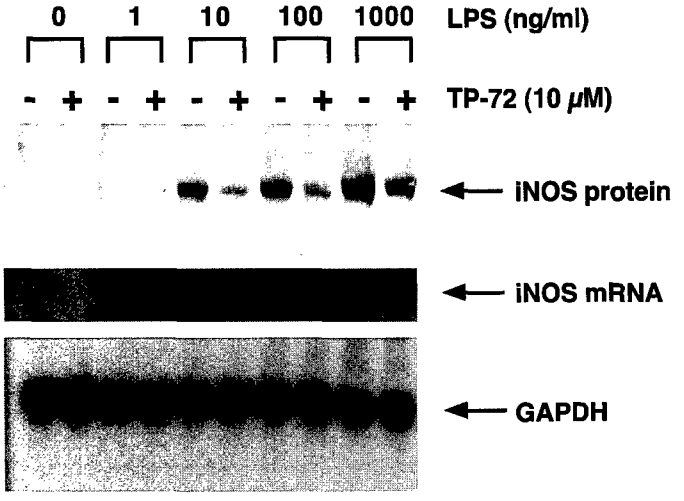


Fig. 3. Inhibition of expression of iNOS protein and mRNA by TP-72 in RAW 264.7 cells. LPS dose-dependently induced iNOS protein and mRNA expression at concentrations of 1, 10, 100, and 1000 ng/ml, and this induction was inhibited by TP-72 (10 μM). LPS and TP-72 were applied simultaneously, and cells were harvested 18 h later. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

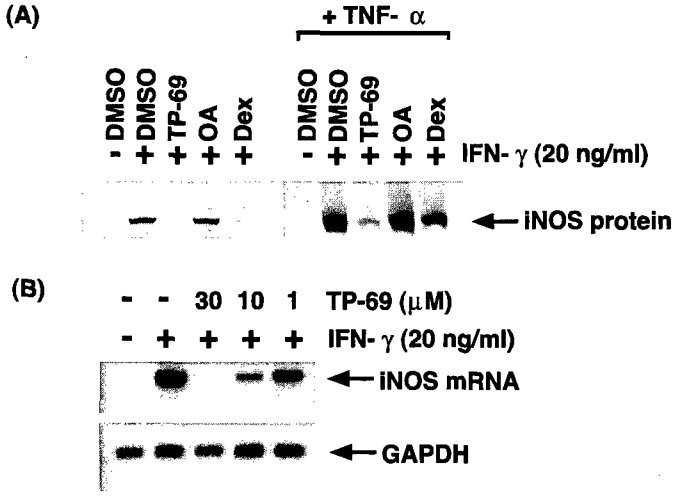


Fig. 4. Inhibition of iNOS protein and mRNA expression by triterpenoids in primary mouse macrophages. *A*, suppression of IFN-γ-induced iNOS protein expression by TP-69. Primary mouse macrophages were treated with IFN-γ (20 ng/ml) or with IFN-γ plus TNF-α (100 ng/ml) for 18 h and then harvested to obtain whole cell lysates. TP-69 (10 μM), OA (10 μM), and dexamethasone (*Dex*; 1 μM) were added simultaneously with IFN-γ or TNF-α. *B*, inhibition of IFN-γ-induced iNOS mRNA expression by TP-69. RNA samples were obtained from IFN-γ-treated primary macrophages after 18 h of incubation.

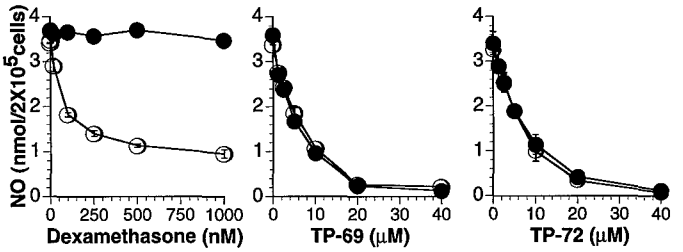


Fig. 5. Blockage by glucocorticoid antagonist RU486 of dexamethasone-inhibited NO production in macrophages but not of triterpenoid-inhibited NO production in macrophages. RAW 264.7 cells were incubated with LPS (10 ng/ml) together with dexamethasone or triterpenoids without RU486 (○); in some cases, RU486 (1 μM) was added simultaneously to both dexamethasone- and triterpenoid-treated cell wells (●). Duration of experiment was 18 h. RU486 itself does not interfere with NO production at the concentration tested.

Triterpenoids Decrease Inducible COX-2 mRNA and Protein Levels, but not Constitutive COX-1, in Macrophages. LPS-induced COX-2 protein (M_r ≈72,000) and COX-2 mRNA (4.4 kb) in RAW 264.7 cells in a dose-dependent manner. As shown in Fig. 6*A*, COX-2 protein and mRNA expression induced by LPS (at concentrations ranging from 1–1000 ng/ml) was markedly decreased by concomitant treatment with TP-72 (10 μM). Fig. 6*B* shows that derivatives of both OA and UA have inhibitory effects on COX-2 protein expression. In addition to TP-72, the oleanane enone, TP-69, also inhibited COX-2 protein expression. The 3-α-epimer of UA, TP-52, suppresses LPS-induced COX-2 protein expression, whereas UA itself does not have an inhibitory effect. The amount of the product of the COX-2 enzyme, PGE_2, in the supernatants from each treatment of the RAW 264.7 cells was determined and corresponded with the COX-2 protein data (Fig. 6*B*). Thus, LPS markedly increased PGE_2 levels, and OA and UA did not substantially affect this increase, whereas TP-69, TP-72, and TP-52 (assayed at 10 μM) all blocked the inductive effect of LPS on the production of PGE_2. However, as shown above for suppression of NO formation, the inhibition of prostaglandin formation by TP-72 is not a result of the inhibition of enzyme activity itself. When this triterpenoid was added to RAW cells in which synthesis of COX-2 had already been induced by LPS, there was no decrease in prostaglandin production, using added arachidonic acid as a substrate (Table 3). COX-1 protein levels were not affected by any of the treatments (Fig. 6*B*).

Triterpenoids Suppress the Activation of NF-κB in Nuclear Extracts from Primary Macrophages. Because activation of NF-κB is critical for the induction of both iNOS and COX-2 by LPS or other inflammatory cytokines (39, 40), we determined whether triterpenoids might suppress NF-κB activation in nuclear extracts obtained from primary macrophages induced with IFN-γ, LPS, or TNF-α in cell cultures. As shown in Fig. 7*A*, TP-72 (20 μM) or dexamethasone (1 μM) inhibited the activation of NF-κB in nuclear extracts obtained from macrophages treated with 10 ng/ml IFN-γ. It should be emphasized that the primary macrophages used in these experiments were elicited *in vivo* by injection of mice with thioglycollate broth, which is known to contain small amounts of LPS (41). Thus, there is a significant basal (preinduced) level of NF-κB in the DMSO control lane (first lane on left) in Fig. 7*A*, resulting from the

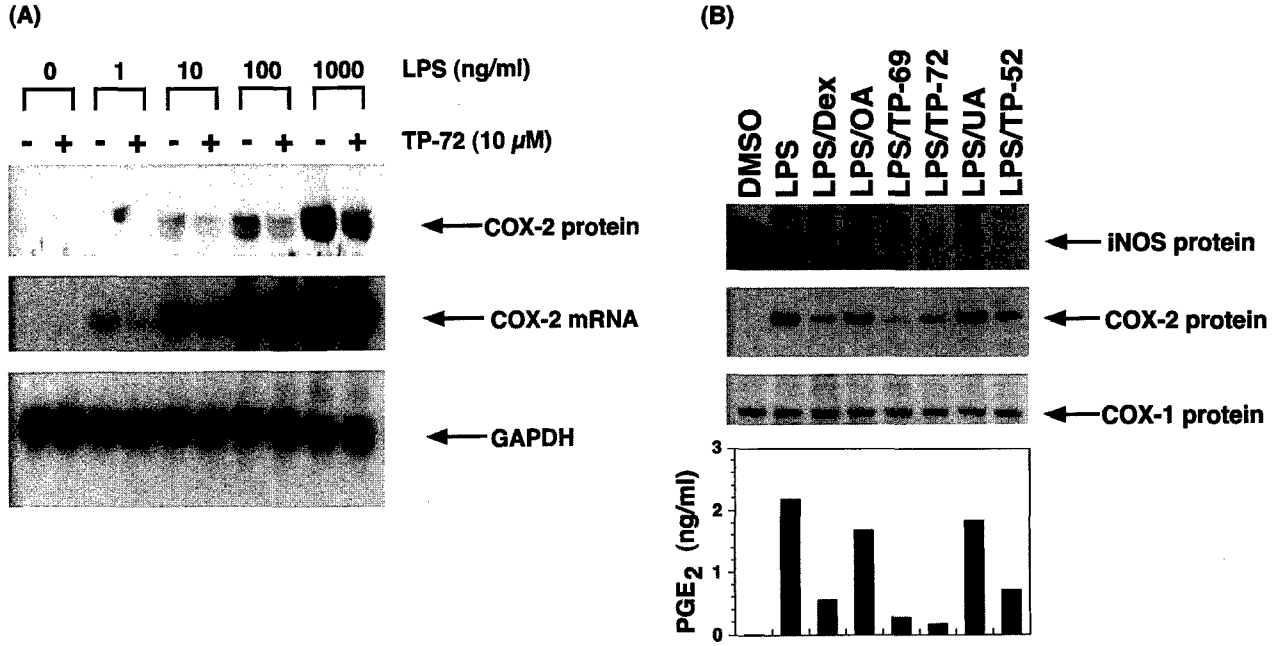


Fig. 6. Inhibition of COX-2 protein and mRNA expression by triterpenoids in RAW 264.7 cells. *A*, LPS dose-dependently induced COX-2 protein and mRNA expression over the ranges of 1, 10, 100, and 1000 ng/ml, and this was suppressed by TP-72 (10 μM). RNA samples were obtained from LPS-treated RAW 264.7 cells after 18 h treatment. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *B*, RAW 264.7 cells were treated with compounds and LPS (10 ng/ml) for 6 h. All of the triterpenoids were tested at 10 μM, and dexamethasone (*Dex*) was at 1 μM. The amount of PGE_2 from the supernatant was determined by PGE_2 kit.

use of thioglycollate (41). The priming of these primary mouse macrophages by the suboptimal levels of LPS present in thioglycollate allows for the further induction by IFN-γ shown in Fig. 7*A*. TP-69 also attenuated activation of NF-κB in primary mouse macrophages (data not shown). To confirm the NF-κB binding proteins in the retarded complexes, antibodies specific to either the p65 or p50 subunits of NF-κB were used to demonstrate the retardation of NF-κB. The mobility of bands was further retarded, particularly by antibody to p65. The data in Fig. 7*B* show that TP-72 (20 μM) inhibited the activation of NF-κB in nuclear extracts obtained from primary macrophages treated with either 10 ng/ml IFN-γ alone (80% inhibition) or by IFN-γ in combination with 10 ng/ml LPS (50% inhibition) or 10 ng/ml TNF-α (70% inhibition).

DISCUSSION

OA and UA have significant, although relatively weak, anti-inflammatory and anticarcinogenic actions, particularly *in vivo* (26, 28, 29, 42). However, there has been a paucity of convincing data from cell culture experiments relating to the mechanism of action of OA and UA. It is possible that OA and UA are precursors to more active molecules that are formed by metabolism, as is the case for dietary vitamins A and D. The experiments reported here now show that synthetic triterpenoids are markedly more active than the parent structures. Among some 80 derivatives we have made, we have found several molecules, namely, 3-epi-ursolic acid (TP-52), 3,12-dioxoolean-1-en-28-oic acid (TP-69), and 3,11-dioxoolean-1,12-dien-28-oic acid (TP-72), which are significantly more active than OA and UA in suppression of the formation of either NO or prostaglandins. We assayed these new synthetic agents as potential suppressors of iNOS or COX-2 because of the highly relevant nature of these two enzymes for many disease processes. Although numerous agents have been synthesized that are effective inhibitors by acting as substrate analogues for each of these two enzymes, an alternative approach to their control, to block *de novo* enzyme formation selectively, has been essentially unexplored.

Table 3 *Effect of added triterpenoid or dexamethasone after LPS induction of COX-2 enzyme in RAW 264.7 cells*

LPS pretreatment of cells[a]	Addition to LPS-treated RAW cells	PGE_2 (ng/ml)[b]
None	DMSO control	0.1 ± 0.0
LPS (10 ng/ml), 6 h	DMSO control	3.4 ± 0.1
	Indomethacin (20 μM)	0.2 ± 0.1[c]
	Dexamethasone (2 μM)	3.4 ± 0.2
	TP-72 (20 μM)	3.5 ± 0.1

[a] RAW 264.7 cells were stimulated for 6 h with LPS (10 ng/ml), and cells were washed twice with fresh medium. Indomethacin, TP-72, or dexamethasone was then added and equilibrated for 30 min. The cells were further incubated with arachidonic acid (100 μM) for 15 min.

[b] The amount of PGE_2 in the supernatant was assayed as described above. Data are means ± SE of four samples from two independent experiments. In each experiment, duplicate determinations were made for each treatment.

[c] $P < 0.001$, significantly different from LPS alone, analyzed by Student's *t* test.

The anti-inflammatory and anticarcinogenic activities of the naturally occurring triterpenoids are relatively weak. Much more potent synthetic analogues are needed if this class of compounds is to be of clinical value. The two synthetic enone derivatives of OA, TP-69 and TP-72, represent a first effort in this direction. Both are highly active in suppressing expression of both iNOS and COX-2 mRNA and protein at concentrations at which their parent molecule, OA, is inactive. TP-69 and TP-72 exert parallel effects on suppression of the expression of both iNOS and COX-2, suggesting that there may be a common mechanistic basis for this action. Suppression of activation of NF-κB by active triterpenoids may partially account for this, because there are known to be NF-κB response elements on the promoters for both the *iNOS* and the *COX-2* genes (40, 43–47). However, not all genetic regulation of the iNOS or COX-2 systems is transcriptional. It has been shown that both TGF-β and dexamethasone may have potent inhibitory effects on the stability or translatability of iNOS or COX-2 mRNAs (21, 23, 48). Some of the effects of the triterpenoids may be mediated at these levels rather than by a direct effect on transcription itself.

Although glucocorticoids block the induction of iNOS and COX-2, they are limited in their usefulness for therapy of chronic disease

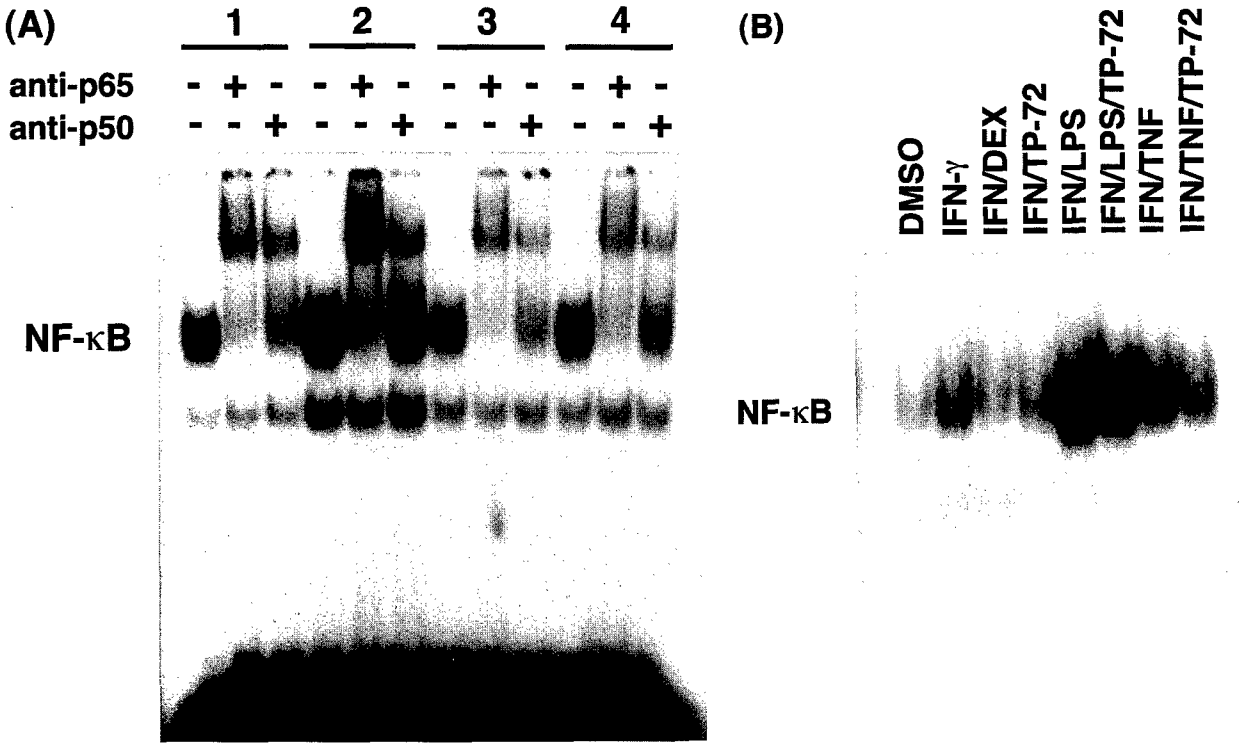


Fig. 7. Suppression of NF-κB by triterpenoids in nuclear extracts from mouse macrophages. Electromobility shift assay using a 5′-end-labeled consensus oligonucleotide was performed with nuclear extracts prepared from primary mouse macrophages, elicited *in vivo* by injection with thioglycollate broth, which is known to contain small amounts of LPS (41). *A,* macrophages were pretreated with compounds for 1 h, and then IFN-γ (10 ng/ml) was added for 1 h. Antibodies to either p65 (anti-p65) or p50 (anti-50) were added to the incubation mixtures for an additional hour. *1,* DMSO; *2,* IFN-γ (10 ng/ml); *3,* IFN-γ + dexamethasone (1 μM); *4,* IFN-γ + TP-72 (20 μM). *B,* macrophages were pretreated with compounds for 1 h (dexamethasone, 1 μM; TP-72, 20 μM), and then IFN-γ (10 ng/ml), TNF-α (10 ng/ml), and LPS (10 ng/ml) were added for 1 h further before making nuclear extracts. *Dex,* dexamethasone; *TNF,* tumor necrosis factor.

states because of the side effects resulting from activation of the glucocorticoid receptor. In contrast, our data suggest that triterpenoids exert their effects through a receptor system other than the glucocorticoid receptor. The nature of the putative triterpenoid receptor remains to be defined. The steroid-like structure and activity of triterpenoids indicates that such a receptor might have some relationship to the steroid receptor superfamily. The striking difference that we have shown between UA (3-β-OH) and its 3-α-hydroxy epimer (TP-52) in their ability to suppress COX-2 synthesis provides particularly strong evidence for receptor-mediated activity, because these two epimers differ only with respect to the conformation of a hydroxyl group (equatorial or axial, respectively) in an essentially planar ring system.

Recently, there have been striking advances that indicate that overexpression of either iNOS and COX-2 may be intimately involved in the pathogenesis of many common debilitating or fatal chronic diseases. These include colon cancer (4, 18, 19, 49), multiple sclerosis (15, 50), Parkinson's disease (16, 51), and Alzheimer's disease (52, 53). There is intense effort to develop enzyme inhibitors that are selective for the inducible forms of these enzymes and do not affect the desirable activity of their respective constitutive isoforms. To the extent that the pathogenesis of the above diseases is promoted by excessive production of NO or prostaglandins, such selective enzyme inhibitors are promising agents for therapy (54). In addition to these specific inhibitors of the inducible enzymes, the possibility of selective repression of inducible enzyme formation also needs to be considered, and triterpenoids now offer one new approach to this mechanism. The combination of inhibition of inducible enzyme formation, together with selective inhibition of inducible enzyme activity, may provide the most effective therapeutic approach. Clearly, more effort needs to be devoted to the chemistry and biology of triterpenoids, especially to develop a more systematic understanding of structure-activity relationships, which at present are only poorly understood (30), and to elucidate the nature of putative receptors for these molecules.

ACKNOWLEDGMENTS

We thank Andrew Dannenberg for suggestions for experiments on COX-2, Harvey Herschman for providing COX-1 and COX-2 cDNA probes, Jan Vilcek for TNF-α, Allan Munck for advice on the use of RU486, and Anita Roberts for comments on the manuscript. M. B. S. is Oscar M. Cohn Professor and is indebted to Allan Conney for an introduction to the pharmacology of UA and OA.

REFERENCES

1. Hong, W. K., and Sporn, M. B. Recent advances in chemoprevention of cancer. Science (Washington DC), *278:* 1073–1077, 1997.
2. Sporn, M. B., and Roberts, A. B. Peptide growth factors and inflammation, tissue repair, and cancer. J. Clin. Invest., *78:* 329–332, 1986.
3. Ohshima, H., and Bartsch, H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutat. Res., *305:* 253–264, 1994.
4. Marnett, L. J. Aspirin and the potential role of prostaglandins in colon cancer. Cancer Res., *52:* 5575–5589, 1992.
5. Moncada, S., Palmer, R. M. J., and Higgs, E. A. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev., *43:* 109–141, 1991.
6. Anggard, E. Nitric oxide: mediator, murderer, and medicine. Lancet, *343:* 1199–1206, 1994.
7. Nathan, C. F., and Xie, Q. W. Nitric oxide synthases: roles, tolls, and controls. Cell, *78:* 915–918, 1994.
8. Seibert, K., and Masferrer, J. Role of inducible cyclooxygenase (COX-2) in inflammation. Receptor, *94:* 17–23, 1994.
9. Salvemini, D., Seibert, K., Masferrer, J. L., Misko, T. P., Currie, M. G., and Needleman, P. Endogenous nitric oxide enhances prostaglandin production in a model of renal inflammation. J. Clin. Invest., *93:* 1940–1947, 1994.
10. Tamir, S., and Tannenbaum, S. R. The role of nitric oxide (NO) in the carcinogenic process. Biochim. Biophys. Acta, *1288:* F31–F36, 1996.
11. Chhatwal, V. J. S., Ngoi, S. S., Chan, S. T. F., Chia, Y. W., and Moochhala, S. M.

Aberrant expression of nitric oxide synthase in human polyps, neoplastic colonic mucosa and surrounding peritumoral normal mucosa. Carcinogenesis (Lond.), *15:* 2081–2085, 1994.
12. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res., *55:* 3785–3789, 1995.
13. Eberhart, C. E., and DuBois, R. N. Eicosanoids and the gastrointestinal tract. Gastroenterology, *109:* 285–301, 1995.
14. Cobbs, C. S., Brenman, J. E., Aldape, K. D., Bredt, D. S., and Israel, M. A. Expression of nitric oxide synthase in human central nervous system tumors. Cancer Res., *55:* 727–730, 1995.
15. Misko, T. P., Trotter, J. L., and Cross, A. H. Mediation of inflammation by encephalitogenic cells: interferon γ induction of nitric oxide synthase and cyclooxygenase 2. J. Neuroimmunol., *61:* 195–204, 1995.
16. Simonian, N. A., and Coyle, J. T. Oxidative stress in neurodegenerative diseases. Annu. Rev. Pharmacol. Toxicol., *36:* 83–106, 1996.
17. DuBois, R. N., Radhika, A., Reddy, B. S., and Entingh, A. J. Increased cyclooxygenase-2 levels in carcinogen-induced rat colon tumors. Gastroenterology, *110:* 1259–1262, 1996.
18. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in $Apc^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell, *87:* 803–809, 1996.
19. Takahashi, M., Fukuda, K., Ohata, T., Sugimura, T., and Wakabayashi, K. Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat colon tumors induced by azoxymethane. Cancer Res., *57:* 1233–1237, 1997.
20. O'Banion, M. K., Winn, V. D., and Young, D. A. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. Proc. Natl. Acad. Sci. USA, *89:* 4888–4892, 1992.
21. Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q. W., and Nathan, C. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. J. Exp. Med., *178:* 605–613, 1993.
22. Nathan, C. F., and Xie, Q. W. Regulation of biosynthesis of nitric oxide. J. Biol. Chem., *269:* 13725–13728, 1994.
23. Perrella, M. A., Yoshizumi, M., Fen, Z., Tsai, J. C., Hsieh, C. M., Kourembanas, S., and Lee, M. E. Transforming growth factor-β1, but not dexamethasone, down-regulates nitric oxide synthase mRNA after its induction by interleukin-1β in rat smooth muscle cells. J. Biol. Chem., *269:* 14595–14600, 1994.
24. Reddy, S. T., Gilbert, R. S., Xie, W., Luner, S., and Herschman, H. R. TGF-β1 inhibits both endotoxin-induced prostaglandin synthesis and expression of the TIS10/prostaglandin synthase 2 gene in murine macrophages. J. Leukocyte Biol., *55:* 192–200, 1994.
25. Kunz, D., Walker, G., Eberhardt, W., and Pfeilschifter, J. Molecular mechanisms of dexamethasone inhibition of nitric oxide synthase expression in interleukin 1β-stimulated mesangial cells: evidence for the involvement of transcriptional and posttranscriptional regulation. Proc. Natl. Acad. Sci. USA, *93:* 255–259, 1996.
26. Huang, M. T., Ho, C. T., Wang, Z. Y., Ferraro, T., Lou, Y. R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J. D., and Conney, A. H. Inhibition of skin tumorigenesis by rosemary and its constituents carnesol and ursolic acid. Cancer Res., *54:* 701–708, 1994.
27. Nishino, H., Nishino, A., Takayasu, J., Hasegawa, T., Iwashima, A., Hirabayashi, K., Iwata, S., and Shibata, S. Inhibition of the tumor-promoting action of 12-*O*-tetradecanoylphorbol-13-acetate by some oleanane-type triterpenoid compounds. Cancer Res., *48:* 5210–5215, 1988.
28. Hirota, M., Mori, T., Yoshio, M., and Iriye, R. Suppression of tumor promoter-induced inflammation of mouse ear by ursolic acid and 4,4-dimethylcholestane derivatives. Agric. Biol. Chem., *54:* 1073–1075, 1990.
29. Singh, G. B., Singh, S., Bani, S., Gupta, B. D., and Banerjee, S. K. Anti-inflammatory activity of oleanolic acid in rats and mice. J. Pharm. Pharmacol., *44:* 456–458, 1992.
30. Honda, T., Finlay, H. J., Gribble, G. W., Suh, N., and Sporn, M. B. New enone derivatives of oleanolic acid and ursolic acid as inhibitors of nitric oxide production in mouse macrophages. Bioorg. Med. Chem. Lett., *7:* 1623–1628, 1997.
31. Bogdan, C., Paik, J., Vodovotz, Y., and Nathan, C. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-β and interleukin-10. J. Biol. Chem., *267:* 23301–23308, 1992.
32. Ding, A., Nathan, C. F., Graycar, J., Derynck, R., Stuehr, D. J., and Srimal, S., Macrophage deactivating factor and transforming growth factors-β1, -β2, and -β3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-γ. J. Immunol., *145:* 940–944, 1990.
33. Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science (Washington DC), *256:* 225–228, 1992.
34. Chomczynski, P., and Sacchi, N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.
35. Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., and Needleman, P. Nitric oxide activates cyclooxygenase enzymes. Proc. Natl. Acad. Sci. USA, *90:* 7240–7244, 1993.
36. Barchowsky, A., Dudek, E. J., Treadwell, M. D., and Wetterhahn, K. E. Arsenic induces oxidant stress and NF-κB activation in cultured aortic endothelial cells. Free Radical Biol. Med., *21:* 783–790, 1996.
37. Masferrer, J. L., Zweifel, B. S., Seibert, K., and Needleman, P. Selective regulation of cellular cyclooxygenase by dexamethasone and endotoxin in mice. J. Clin. Invest., *86:* 1375–1379, 1990.
38. Gilbert, R. S., and Herschman, H. R. "Macrophage" nitric oxide synthase is a glucocorticoid-inhibitable primary response gene in 3T3 cells. J. Cell. Physiol., *157:* 128–132, 1993.
39. Collart, M. A., Baeuerle, P., and Vassalli, P. Regulation of tumor necrosis factor α transcription in macrophages: involvement of four κB-like motifs and of constitutive and inducible forms of NF-κB. Mol. Cell. Biol., *10:* 1498–1506, 1990.
40. Xie, Q. W., Kashiwabara, Y., and Nathan, C. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J. Biol. Chem., *269:* 4705–4708, 1994.
41. Jin, F-y., Nathan, C., Radzioch, D., and Ding, A. Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. Cell, *88:* 417–426, 1997.
42. Kapil, A., and Sharma, S. Effect of oleanolic acid on complement in adjuvant- and carrageenan-induced inflammation in rats. J. Pharm. Pharmacol., *47:* 585–587, 1995.
43. Xie, W., Merrill, J. R., Bradshaw, W. S., and Simmons, D. L. Structural determination and promoter analysis of the chicken mitogen-inducible prostaglandin G/H synthase gene and genetic mapping of the murine homolog. Arch. Biochem. Biophys., *300:* 247–252, 1993.
44. Roshak, A. K., Jackson, J. R., McGough, K., Chabot-Fletcher, M., Mochan, E., and Marshall, L. A. Manipulation of distinct NFκB proteins alters interleukin-1β-induced human rheumatoid synovial fibroblast prostaglandin E2 formation. J. Biol. Chem., *271: 31496–31501, 1996.*
45. Barnes, P. J., and Karin, M. Nuclear factor-κB–a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med., *336:* 1066–1071, 1997.
46. Crofford, L. J., Tan, B., McCarthy, C. J., and Hla, T. Involvement of nuclear factor κB in the regulation of cyclooxygenase-2 expression by interleukin-1 in rheumatoid synoviocytes. Arthritis Rheumat., *40:* 226–236, 1997.
47. Schmedtje, J. F., Jr., Ji, Y. S., Liu, W. L., DuBois, R. N., and Runge, M. S. Hypoxia induces cyclooxygenase-2 via the NF-κB p65 transcription factor in human vascular endothelial cells. J. Biol. Chem., *272:* 601–608, 1997.
48. Radomski, M. W., Palmer, R. M., and Moncada, S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc. Natl. Acad. Sci. USA, *87:* 10043–10047, 1990.
49. Prescott, S. M., and White, R. L. Self-promotion? Intimate connections between APC and prostaglandin H synthase-2. Cell, *87:* 783–786, 1996.
50. Hooper, D. G., Bagasra, O., Marin, J. C., Zborek, A., Ohnishi, S. T., Kean, R., Champion, J. M., Sarker, A. B., Bobroski, L., Farber, J. L., Akaike, T., Maeda, H., and Koprowski, H. Prevention of experimental allergic encephalomyelitis by targeting nitric oxide and peroxynitrite: implications for the treatment of multiple sclerosis. Proc. Natl. Acad. Sci. USA, *94:* 2528–2533, 1997.
51. Hantraye, P., Brouillet, E., Ferrante, R., Palfi, S., Dolan, R., Matthews, R. T., and Beal, M. F. Inhibition of neuronal nitric oxide synthase prevents MPTP-induced Parkinsonism in baboons. Nat. Med., *2:* 1017–1021, 1996.
52. Goodwin, J. L., Uemura, E., and Cunnick, J. E. Microglial release of nitric oxide by the synergistic action of β-amyloid and IFN-γ. Brain Res., *692:* 207–214, 1995.
53. Good, P. F., Werner, P., Hsu, A., Olanow, C. W., and Perl, D. P. Evidence for neuronal oxidative damage in Alzheimer's disease. Am. J. Pathol., *149:* 21–28, 1996.
54. Frölich, J. C. A classification of NSAIDs according to the relative inhibition of cyclooxygenase isoenzymes. Trends Pharmacol. Sci., *18:* 30–34, 1997.

Bio-Organic and Medicinal Chemistry Letters (In Press)

DESIGN AND SYNTHESIS OF 2-CYANO-3,12-DIOXOOLEAN-1,9-DIEN-28-OIC ACID, A NOVEL AND HIGHLY ACTIVE INHIBITOR OF NITRIC OXIDE PRODUCTION IN MOUSE MACROPHAGES

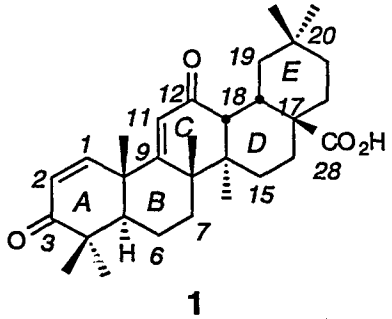
Tadashi Honda,[a] BarbieAnn V. Rounds,[a] Gordon W. Gribble,*[,a]
Nanjoo Suh,[b] Yongping Wang,[b] and Michael B. Sporn*[,b]

[a]*Department of Chemistry, Dartmouth College, Hanover, NH 03755, U.S.A. and*
[b]*Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755, U.S.A.*

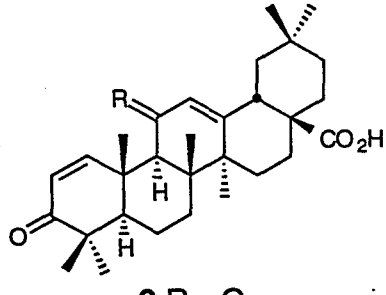
Abstract: New derivatives with electron-withdrawing substituents at the C-2 position of 3-oxoolean-1-en-28-oic acid were synthesized. Among them, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) was 400 times more potent than previous compounds we have made as an inhibitor of production of nitric oxide induced by interferon-γ in mouse macrophages (IC_{50}, 0.4 nM). The potency of CDDO was similar to that of dexamethasone, although CDDO does not act through the glucocorticoid receptor.

Introduction

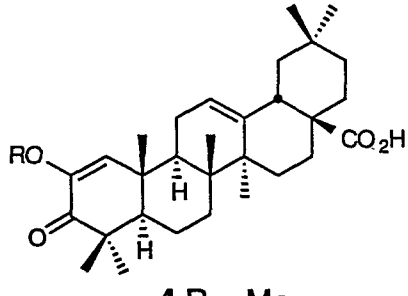
In a previous communication we reported that oleanolic acid derivatives with a 1-en-3-one functionality in ring A (e.g., **1**–**3**) have significant inhibitory activity against production of nitric oxide (NO) induced by interferon-γ (IFN-γ) in mouse macrophages (IC_{50}, 0.1–1 μM). We also showed that derivatives with electron-releasing substituents at the C-2 position, **4** and **5**, lose the activity.[1] Mechanism studies showed that enones **1** and **2** suppress transcription or translation of the inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) genes, and that these compounds do not act through a glucocorticoid receptor.[2] We therefore focused on the design and synthesis of derivatives with electron-withdrawing substituents at the C-2 position to obtain more active compounds. We have now found that 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) (**6**) has strong activity (IC_{50}, 0.4 nM), with a potency similar to that of dexamethasone. In this communication, the design, synthesis, and inhibitory activity are reported for these compounds.



1



2 R = O
3 R = H_2

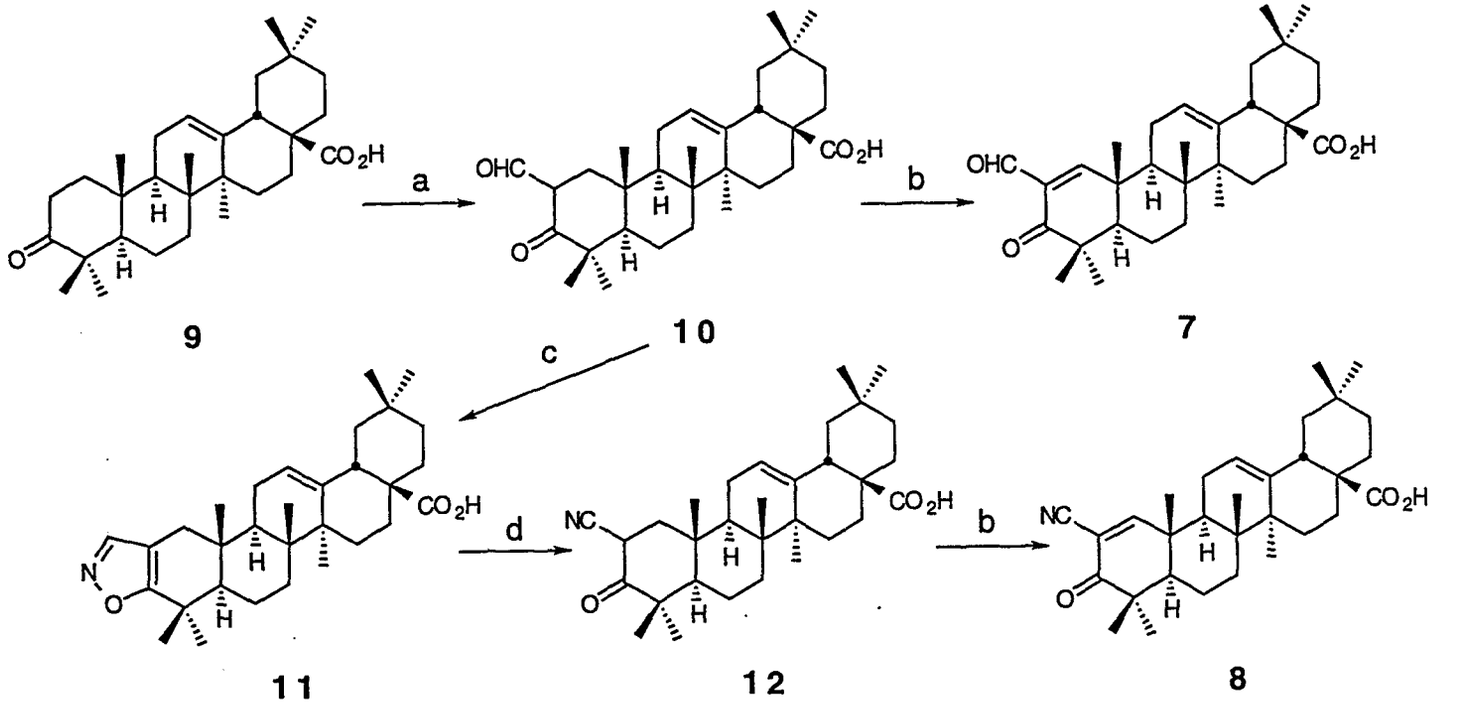


4 R = Me
5 R = H

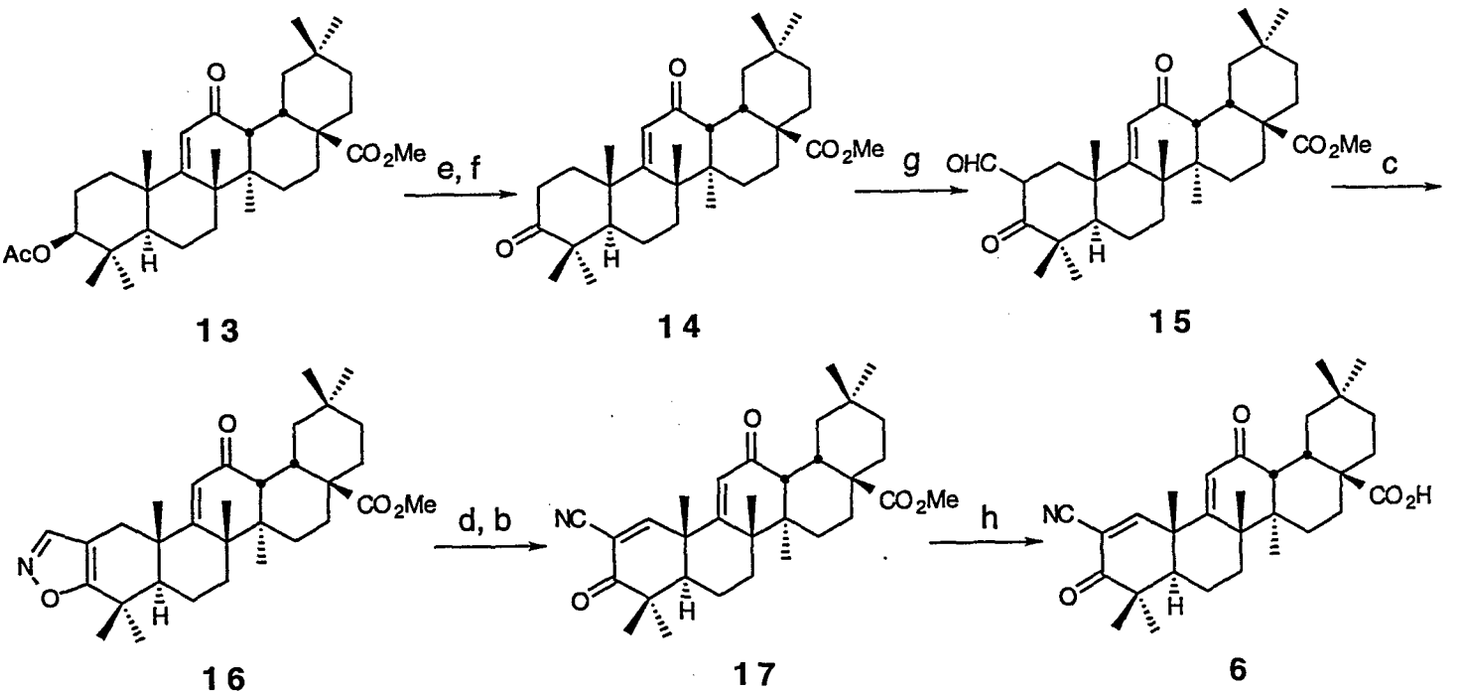
Design and Synthesis of New Derivatives

Initially, compounds **7** and **8** were synthesized according to the route illustrated in Scheme 1. Compound **10** was prepared by formylation of oleanonic acid (**9**)[3] with ethyl formate in the presence of sodium methoxide in

Scheme 1.



Scheme 2.



a: HCO_2Et / MeONa / THF, b: PhSeCl / AcOEt; 30%H_2O_2 / THF, c: NH_2OH·HCl / EtOH / H_2O,
d: MeONa / MeOH / Et_2O, e: KOH / MeOH, f: Jones, g: HCO_2Et / MeONa / PhH, h: LiI / DMF

THF[4] [yield, 45% (66% based on recovered **9**)]. Aldehyde **7** was obtained in 29% yield by introduction of a double bond at C-1 of **10** with phenylselenenyl chloride in ethyl acetate and sequential addition of 30% hydrogen peroxide[5] ($PhSeCl-H_2O_2$). Nitrile **12** was synthesized via isoxazole **11** from **10** according to Johnson's method.[6] Isoxazole **11** was synthesized in 99% yield from **10** by addition of hydroxylamine in aqueous ethanol.

Cleavage of isoxazole **11** with sodium methoxide gave nitrile **12** in 98% yield. Compound **8** was obtained in 36% yield by introduction of a double bond at C-1 of **12** with PhSeCl-H_2O_2. Compound **7** was toxic to cells in culture. Compound **8** was more potent than **3** (see Table). We therefore designed the new target **6** based on both structures of **1** and **8**, because **1** is also much more active than **3** (see Table and ref 1). The synthesis of **6** is illustrated in Scheme 2. Compound **14** was prepared in 89% yield from known compound **13**[7] by alkali hydrolysis, followed by Jones oxidation. Compound **15** was prepared in quantitative yield by formylation of **14** with ethyl formate in the presence of sodium methoxide in benzene. Isoxazole **16** was synthesized in 61% yield from **15** by the addition of hydroxylamine. Nitrile **17** was obtained by cleavage of isoxazole **16** with sodium methoxide (yield, 100%), followed by introduction of a double bond at C-1 with PhSeCl-H_2O_2 (yield, 40%). CDDO (**6**) was prepared in 71% yield by halogenolysis of **17** with lithium iodide in DMF.[8]

Biological Results and Discussion

The inhibitory activities [IC_{50} (μM) value] of compounds **1–8**,[9] oleanolic acid, and dexamethasone (a positive control) on production of NO induced by IFN-γ in mouse macrophages[10] are shown in the Table. Compound **8** was more active than **3** but less active than **1**. CDDO (**6**) was a strong inhibitor (IC_{50}, 0.4 nM), equivalent to dexamethasone. However, the inhibitory activity of **6** was not blocked by the glucocorticoid antagonist, RU-486,[11] which reverses the action of dexamethasone.

Table. IC_{50} (μM)[a] Values for Inhibition of Production of NO Induced by IFN-γ in Mouse Macrophages[10]

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
dexamethasone	0.0003	**5**	37
1	0.17	CDDO (**6**)	0.0004
2	1.4	**7**	> 1[b]
3	7.1	**8**	0.6
4	19	oleanolic acid	> 40

[a]IC_{50} (μM) values of compounds **1–5**, **7** and **8** were determined in the range of 0.01–40 μM (4-fold dilutions); dexamethasone and **6** were assayed in the range of 0.1 pM–1 μM (10-fold dilutions). Values are an average of two separate experiments.
[b]Compound **7** was toxic to cells above 1 μM and was not active below 1 μM.

These results provide the following interesting structure–activity relationships:

(1) A nitrile group at C-2 enhances activity. Compounds **6** and **8** are more potent than **1** and **3**, respectively.

(2) Hydroxyl and methoxy groups at C-2 decrease activity. Compounds **4** and **5** were much less potent than **3**.

(3) The above results suggest that electron-withdrawing groups at C-2 increase potency, and electron-releasing groups decrease potency.

(4) A 9-en-12-one functionality is also a strong enhancer of potency. Compounds **1** and **6** are more active than **3** and **8**, respectively.

(5) The combination of a 9-en-12-one functionality, together with a nitrile group at C-2, provides a particularly potent compound for suppression of production of NO.

On the basis of these structure–activity relationships, further lead optimization is in progress. Further biological evaluation of CDDO (**6**) is also in progress.[12]

Acknowledgments

We thank Drs. Carl Nathan and Qiao-wen Xie for expert advice on the preparation of macrophages and the nitric oxide assay. We also thank Dr. Mary K. Young and Mr. Ron New (UC Riverside) for the mass spectra, and Professor David A. Evans and Mr. Brett D. Allison (Harvard University) for the optical rotation measurements. This investigation was supported by funds from the Norris Cotton Cancer Center, U.S. Dept. of Defense Grant # DAMD17-96-1-6163, and the Oliver and Jennie Donaldson Charitable Trust. M.B.S. is Oscar M. Cohn Professor, and Y.W. is a Howard Hughes Medical Institute Predoctoral Fellow.

References and Notes

1. Honda, T.; Finlay, H. J.; Gribble, G. W.; Suh, N.; Sporn, M. B. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1623.
2. Suh, N.; Honda, T.; Finlay, H. J.; Barchowsky, A.; Williams, C.; Benoit, N. E.; Xie, Q.; Nathan, C.; Gribble, G. W.; Sporn, M. B. *Cancer Res.* **1998**, *58*, 717. (b) Suh, N.; Williams, C.; Xie, Q.; Nathan, C.; Honda, T.; Finlay, H. J.; Gribble, G. W.; Sporn, M. B. *Proceedings of the 89th Annual Meeting of American Association for Cancer Research*, New Orleans, LA, **1998**,
3. Simonsen, J.; Ross, W. C. J. In *The Terpenes*; Cambridge University: Cambridge, 1957; Vol 5, pp 221–285.
4. Clinton, R. O.; Manson, A. J.; Stonner, F. W.; Neumann, H. C.; Christiansen, R. G.; Clarke, R. L.; Ackerman, J. H.; Page, D. F.; Dean, J. W.; Dickinson W. B.; Carabateas, C. *J. Am. Chem. Soc.* **1961**, *83*, 1478.
5. Sharpless, K. B.; Lauer, R. F.; Teranishi, A. Y. *J. Am. Chem. Soc.* **1973**, *95*, 6137.
6. Johnson, W. S.; Shelberg, W. E. *J. Am. Chem. Soc.* **1945**, *67*, 1745.
7. Picard, C. W.; Sharples, K. S.; Spring, F. S. *J. Chem. Soc.* **1939**, 1045.
8. Dean, P. D. G. *J. Chem. Soc.* **1965**, 6655.
9. All new compounds **6–8** exhibited satisfactory spectral data including high-resolution mass spectra and elemental analyses. CDDO (**6**): amorphous solid; $[\alpha]^{22}_{D}$ +33 ° (*c* 0.28, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 240.4 (4.21) nm; IR (KBr) 2950, 2867, 2235, 1692, 1665 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.05 (1H, s), 5.99 (1H, s), 3.10-3.00 (2H, m), 1.49, 1.35, 1.26, 1.17, 1.02, 1.00, 0.91 (each 3H, s); ^{13}C NMR ($CDCl_3$) δ 199.0, 196.8, 183.6, 168.8, 166.0, 124.3, 114.9, 114.6, 50.0, 47.9, 47.2, 46.0, 45.3, 42.8, 42.4, 35.9, 34.7, 33.5, 33.1, 31.9, 31.7, 30.9, 28.2, 27.2, 26.9, 24.9, 23.3, 22.7, 21.8, 18.5; EIMS (70 eV) *m/z* 491 $[M]^+$ (100), 476 (62), 445 (29), 430 (27), 269 (94). HREIMS Calcd for $C_{31}H_{41}NO_4$: 491.3036; Found: 491.3020. Anal. Calcd for $C_{31}H_{41}NO_4 \cdot 1/4H_2O$ C, 75.04; H, 8.43. Found: C, 75.29; H, 8.79.
10. Briefly, the procedure for this assay is as follows: Macrophages were harvested from female mice injected intraperitoneally four days previously with 4% thioglycollate. These cells were seeded in 96-well tissue culture plates and incubated with 4 ng/mL IFN-γ in the presence or absence of inhibitory test compounds. After 48 hours NO production (measured as nitrite by the Griess reaction) was determined. Full details of the assay are given in reference 13.
11. Gagne, D.; Pons, M.; Philibert, D. *J. Steroid Biochem.* **1985**, *23*, 247.
12. Detailed biological data will be published elsewhere.
13. Ding, A.; Nathan, C.; Graycar, J.; Derynck, R.; Stuehr, D. J.; Srimal, S. *J. Immunol.* **1990**, *145*, 940. (b) Bogdan, C.; Paik, J.; Vodovotz, Y.; Nathan, C. F. *J. Biol. Chem.* **1992**, *267*, 23301.